BIOPHYSICAL CHARACTERIZATION OF ZEBRAFISH TRANSIENT RECEPTOR
POTENTIAL CATION CHANNEL, SUBFAMILY M, MEMBER 7.

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY
OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES (CELL AND MOLECULAR BIOLOGY)

AUGUST 2015

By

Chad Jansen

Dissertation Committee:
Andrea Fleig, Chairperson
Robert Nichols
Frederick Bellinger
Olivier Le Saux
Andrew Stenger
Acknowledgements

Dr. Andrea Fleig

Dr. Robert Nichols

Dr. Frederick Bellinger

Dr. Olivier Le Saux

Dr. Andrew Stenger

Lori Tsue, Chris Maggio, Dr. Reinhold Penner, Dr. Malika Faouzi, Dr. Mahealani Monteilh-Zoller, Dr. Andrew Scharenburg, Dr. Jaya Sahni

The Queen’s Center for Biomedical Research

The Queens Medical Center

NIH Grant: P01GM078195

Hawaii Community Foundation grant: George F. Straub Trust and Ingeborg v.F. McKee Fund

12P-51368

The Department of Cell and Molecular Biology, University of Hawai‘i at Manoa

Natalie Chounet
Abstract

TRPM7 is a non-selective divalent ion channel that belongs to the transient receptor potential superfamily; it is permeable to divalent ions such as calcium and magnesium as well as trace elements such as manganese and zinc. Although TRPM7 is permeable to many divalent ions, it is regulated by intracellular magnesium and magnesium adenosine triphosphate. Moreover, TRPM7 is thought to be involved in magnesium homeostasis of the cell. Magnesium is important for many processes within an organism. When serum levels of magnesium drop, a state of hypomagnesemia is reached. Hypomagnesemia is associated with many symptoms such as tremors, convulsions and even coma. TRPM7 has been implicated in hypomagnesemia, as well as breast cancer metastasis and delayed neuronal death post brain ischemia.

Human TRPM7 has been characterized and studied, but limitations do exist within animal models to study TRPM7. Global knockout of TRPM7 in mice leads to death at embryonic day 7.5. This fact limits the developmental studies that can be performed on a mouse. A non-mammalian model, zebrafish, may be able to bridge the gap in studies of TRPM7. There exists zebrafish with a loss-of-function mutation in TRPM7 that survive; these mutants show skeletogenesis defects and kidney stone formation. Zebrafish TRPM7 has yet to be biophysically characterized. Here, it is investigated whether zebrafish TRPM7 is biophysically similar to human TRPM7. Zebrafish TRPM7 and three truncated mutants of zebrafish TRPM7 were overexpressed in human embryonic kidney cells. The truncated mutants of TRPM7 were utilized as a means to narrow down the magnesium and magnesium adenosine triphosphate binding sites within zebrafish TRPM7.
This study shows that: 1- Zebrafish TRPM7 is biophysically similar to human TRPM7 in regards to regulation, divalent ion permeability and pH sensitivity, 2- Zebrafish TRPM7 is less sensitive to osmolarity than human TRPM7, but remains sensitive. 3- Common human TRPM7 inhibitors do not inhibit Zebrafish TRPM7. Additionally, investigation of the three truncated zebrafish TRPM7 mutants suggests that there is a third magnesium-binding site. Experimental designs with zebrafish as an animal model for TRPM7 pathologies and diseases should take into account the differences in zebrafish TRPM7 and human TRPM7.
# Table of contents

Acknowledgements .............................................................................................................. ii

Abstract............................................................................................................................. iii

Table of contents ................................................................................................................ v

List of figures....................................................................................................................... ix

List of abbreviations ......................................................................................................... xi

Chapter 1: Introduction ..................................................................................................... 15

Ion channels ....................................................................................................................... 15

The Transient Receptor Potential Superfamily of Ion Channels ...................................... 17

TRPM7 ............................................................................................................................... 21

Hypothesis ......................................................................................................................... 32

Chapter 2: Model Systems and Methodology ................................................................ 34

Model systems ................................................................................................................... 34

Human embryonic kidney cells (HEK293) ........................................................................ 34

Zebrafish liver cells (ZFL) .............................................................................................. 34

Molecular Biology ............................................................................................................. 35
Transformation of bacteria .................................................................35
DNA purification ..................................................................................37
Linearization of 5TO ...........................................................................37
DNA gel electrophoresis ......................................................................38
Cell Culture Methods ........................................................................39
Maintenance of cell culture: .................................................................39
Production of inducible stable expression cell line: ............................39
Protein biochemistry ..........................................................................41
Antibodies .........................................................................................41
Cellular preparation for Western Blot ..................................................41
Western Blot .......................................................................................42
Electrophysiology ...............................................................................45
Patch-Clamp Technique .....................................................................45
Patch Pipettes ...................................................................................48
Whole Cell Patch-Clamp .....................................................................48
Solutions ............................................................................................50
Analysis .................................................................................................................. 51

Chapter 3: Biophysical Characterization of drTRPM7 ........................................... 52

1. Introduction ............................................................................................................ 52

2. Results .................................................................................................................... 55

Mg$^{2+}$ and Mg•ATP sensitivity in drTRPM7 .......................................................... 55

Divalent Ion Permeability of drTRPM7 ................................................................. 57

Pharmacological Profile of drTRPM7 .................................................................... 59

Sensitivity of drTRPM7 to extracellular pH changes ........................................... 61

Sensitivity of drTRPM7 to osmotic changes ......................................................... 61

3. Discussion ............................................................................................................. 63

4. Prospective Experiments ..................................................................................... 66

Chapter 4: Analysis of the putative Mg and Mg-ATP regulatory sites in drTRPM7 through the use of truncation mutants .................................................... 68

1. Introduction ............................................................................................................ 68

2. Results .................................................................................................................... 69

Mg•ATP sensitivity in truncation mutants of drTRPM7 ...................................... 69

Mg$^{2+}$ sensitivity in truncation mutants of drTRPM7 .......................................... 70
3. Discussion .........................................................................................................................74

4. Prospective experiments ..................................................................................................77

Chapter 5: Initial characterization of native drTRPM7 ......................................................78

1. Introduction ...................................................................................................................78

2. Results ............................................................................................................................79

   Mg$^{2+}$ sensitivity in endogenously expressed drTRPM7 ................................................79

   2-APB sensitivity in endogenously expressed drTRPM7 ...............................................81

3. Discussion .......................................................................................................................81

4. Prospective experiments ................................................................................................83

Chapter 6: Conclusions .......................................................................................................84

References: .........................................................................................................................89
List of figures

Figure 1: Phylogenic tree of transient receptor potential superfamily (Huang 2004) ..................19

Figure 2. TRPM7 structure and current voltage relationship .........................................................20

Figure 3: human TRPM7, human TRPM6 and zebrafish TRPM7 alignment ..........................31

Figure 4: The 5TO and 6/TR pcDNA plasmids ............................................................................36

Figure 5: Linearization of drTRPM7 plasmid ..............................................................................38

Figure 6: Immuno blot detection for HA-tag drTRPM7 ..............................................................44

Figure 7: Patch Clamp Configurations: .......................................................................................47

Figure 8: Human TRPM7 and TRPM6 biophysical characteristics summary ......................54

Figure 9: Mg$^{2+}$ and Mg•ATP sensitivity in drTRPM7 ...............................................................56

Figure 10: Divalent profile for drTRPM7. ..................................................................................58

Figure 11: Pharmacological inhibitors of TRPM7 effects on drTRPM7 .................................60

Figure 12: pH and osmolarity sensitivity of drTRPM7 .............................................................62

Figure 13: Wild-type drTRPM7 and truncated mutants of drTRPM7 ....................................68

Figure 14: Mg•ATP dose-response in truncated mutants of drTRPM7 in comparison to wild-type drTRPM7 ........................................................................................................71
Figure 15: Free Mg$^{2+}$ dose-response in truncated mutants of drTRPM7 in comparison to wild-type drTRPM7. .............................................................................................................................................73

Figure 16: Free Mg$^{2+}$ and 2-APB sensitivity in endogenously expressed drTRPM7..................80

Figure 17: Summary of biophysical characteristics of TRPM7 .........................................................85

Figure 18: drTRPM7 three binding site model..............................................................................87
List of abbreviations

AgCl: silver chloride

2-APB: 2-aminoethoxydiphenyl borate

ATP: adenosine triphosphate

BAPTA: 1,2-Bis (2-aminophenoxy) ethane N,N,N’N’–tetraacetic acid

Ca^{2+}: calcium Ion

°C: degree Celsius

CO_{2}: carbon dioxide

DMEM: Dulbecco’s modified eagle medium

drTRPM7: danio rerio transient receptor potential melastatin family seven

EDTA: ethylenediaminetetraacetate

EGF: epidermal growth factor

FBS: fetal bovine serum

HA-tag: human influenza hemagglutinin sequence

HEK293 T-REx: human embryonic kidney cells expressing tet repressor

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
hTRPM7: human transient receptor potential melastatin family seven

Hz: hertz

IC\textsubscript{50}: half maximal inhibitory concentration

I/V: current / voltage relationship

K\textsuperscript{+}: potassium Ion

LB broth: lysogeny broth

Mg\textsuperscript{2+}: magnesium Ion

mM: millimolar

Mg•ATP: magnesium adenosine triphosphate

mmol/L: millimoles per liter

ms: millisecond

mTRPM7: murine transient receptor potential melastatin family seven

mV: millivolt

Na\textsuperscript{+}: sodium Ion

Ω: ohm

pA: picoamp
PBS: phosphate buffered saline
pcDNA: plasmid cytomegalovirus promoter deoxyribonucleic acid
pF: picofarad
PMSF: phenylmethylsulfonyl fluoride
PVDF: polyvinyl difluoride
RNA: ribonucleic acid
TBS: tris buffered saline
TRP: transient receptor potential
TRPA: transient receptor potential ankyrin
TRPC: transient receptor potential canonical
TRPM: transient receptor potential melastatin
TRPML: transient receptor potential mucolipan
TRPP: transient receptor potential polycystin
TRPV: transient receptor potential vanilloid
TRPM6: transient receptor potential melastatin family six
TRPM7: transient receptor potential melastatin family seven
$\text{Zn}^{2+}$: Zinc Ion

ZFL: zebrafish liver cell line
Chapter 1: Introduction

Ion channels

There are many different types of organisms, some are single-celled and others contain multiple specialized cells. The plasma membrane separates the cytosolic contents of the cell from its external milieu. The membrane consists of a phospholipid bilayer arranged such that the hydrophilic heads of the phospholipids face the external milieu and cytosol and the lipophilic tails face each other. The arrangement of lipids maintains a separation between the inside and the outside of the cell from hydrophilic molecules. There are also specialized proteins embedded in the lipid bilayer that allow signal transduction, cell adhesion and transportation of molecules across the plasma membrane.

The plasma membrane is impermeable to ions such as sodium (Na\(^+\)), potassium (K\(^+\)), magnesium (Mg\(^{2+}\)) and calcium (Ca\(^{2+}\)), acting like an insulator against electrical charges. Indeed, the membrane effectively represents a capacitor. The cell requires control over ions to maintain normal function. The cell utilizes ion channels and transporters to control the gradient of ions between the inside of the cell and the outside of the cell as well as storage organelles within the cell. A typical cell will maintain high intracellular concentration of K\(^+\) ions and low concentration of Na\(^+\). In the extracellular milieu these concentrations are reversed, effectively creating an electrochemical gradient across the plasma membrane. A cell with such a distribution of ions will have a resting membrane potential of about -70 mV, as determined by the Nernst equation in the face of a small, resting conductance for K\(^+\).
Ion channels are pore-forming proteins in the plasma membrane. These pores have an open and closed state. Open ion channels allow the passive flow of ions thus generating ionic currents (Hille 2001; Sanchez et al. 1986). The characteristics of a specific ion channel vary and include permeability to specific ions, inhibitors and activators of the channel, mechanisms of gating, and whether or not the channel is voltage-dependent (Minke and Cook 2002; Sperelakis 2001). The rate at which an ion will flow through an ion channel depends on ion channel selectivity, the channel’s open state, the membrane potential, and the relative concentrations of the ion on the inside and outside of the cell.

There are different types of gating for ion channels. One type of ion channel is known as the voltage-gated ion channel. This type of ion channel can be found in nervous tissue. The voltage-gated ion channels are typically permeable to a specific ion, such as sodium, potassium or calcium. Voltage-gated ion channel’s subunit structure consists of six transmembrane spans. Four homologous subunits come together to form the pore. Transmembrane spans five and six contain the pore loop, which forms the pore of the voltage-gated ion channels. The pore loop contains the selectivity filter. The selectivity filter determines the ion channels permeability to an ion, such as, sodium’s permeability in voltage-gated sodium channels. The hallmark characteristic of voltage-gated channels is the ability to respond to changes in transmembrane potential. Transmembrane span four is responsible for sensing the changes in transmembrane potential, which is achieved via repeats of one positively charged amino acid follow by two high hydrophobic amino acids (Sperelakis 2001).

Another type of ion channel is the ligand-gated ion channel. These ion channels can be found in nervous tissue responding to neurotransmitters for fast-acting chemical signaling. The
structure of ligand-gated ion channels differs from voltage gated ion channels. Four trans-membrane spans make up each individual subunit. There are five subunits that come together to form the channel. The pore of the channel is formed by the second trans-membrane span. The second trans-membrane span also determines the ion selectivity of the channel and contains the ligand-binding site on the extracellular portion of the channel. Ligand-gated ion channels can be selective for a specific ion, or non-selective and allow differing ions to flow through the ion channel in the open state. The gating property for ligand-gated channels refers to the conformational change that happens when a ligand is bound to the channel. The conformational change can switch the channel from an open state to a closed state. The term agonist refers to a ligand that binds the channel and changes the channel to open state. Alternatively, an antagonist can bind the channel but that binding does not result in a conformational change that switches the channel to an open state. The time that the channel remains in the open state after an agonist binds the channel is referred to as the open probability. Ligand-gated channels are also susceptible to allosteric modulation via covalent modulations such as phosphorylation and non-covalent modulators such as toxins, which can affect the function of the channel (Sperelakis 2001).

The Transient Receptor Potential Superfamily of Ion Channels

In 1989 the “transient receptor potential” (TRP) gene was cloned from Drosophila (Montell et al. 1989), which led to the discovery of the TRP family of ion channels (Fig. 1). TRP superfamily contains 27 channels in humans. All TRP channels consist of subunits that contain six trans-membrane spans. The cation permeable pore region can be found between trans-membrane spans five and six. Each functional channel contains four identical or similar subunits. The regulation
and modulation of TRP channel activity takes place on the cytoplasmic side of the channel. TRP channels have unique cell sensing functions such as, photoreception, hearing, olfaction, taste and somatosensation. Among these unique cell-sensing functions, some TRP channels also have an important role in cellular homeostasis. The TRP cation channels contain six subfamilies of channels: TRPC or TRP “canonical”, TRPV or TRP “vanilloid”, TRPM or TRP “melastatin”, TRPP or TRP “polycystin”, TRPML or TRP “mucolipan” and TRPA or TRP “ankyrin”. These six subfamilies contain their own channels within the family. The functional TRP channel is a tetramer of the same subunits or similar subunits.
Figure 1: Phylogenetic tree of transient receptor potential superfamily (Huang 2004)
Figure 2. TRPM7 structure and current voltage relationship

A) The structure of a TRPM7 subunit. Displayed is melastatin homology region (MHD), transmembrane spans 1 – 6 (S1- S6), TRP domain (TRP), Coiled coil domain (CCD), alpha kinase. TRPM7 is a non-selective divalent cation channel that conducts divalent ions when external divalent ions are present such as calcium and magnesium. In the absence of external divalent ions TRPM7 conducts monovalent ions as well such as sodium and potassium. B) TRPM7 current voltage relationship displaying outward rectification and reverse potential at 0 mV (Bae et al. 2011).

Within the TRP melastatin (TRPM) family there are eight channels, TRPM1-8. TRPM1 channel is involved in rod functioning of the eyes, mutations in TRPM1 leading to a form of night blindness (Li et al. 2009). TRPM2 is a calcium, magnesium and monovalent permeable channel that is gated by ADPR and located in the central nervous system. TRPM2 is thought to be involved in calcium-induced excitotoxicity and delayed neuronal death due to TRPM2 being activated by reactive oxygen species (Faouzi et al. 2014; Xie et al. 2010). TRPM3 is a widely expressed calcium permeable channel that senses heat (Vriens et al. 2011). TRPM4 is a calcium
activated non-selective cation channel. TRPM4 knockout in mouse leads to hypertension as well as provides some protection from experimental allergic encephalomyelitis (a mouse model of multiple sclerosis) (Mathar et al. 2014). TRPM5 is a calcium-activated cation channel that functions in taste sensation as well as insulin secretion in β-cells (Liman 2014). TRPM8 is a sodium and calcium permeable channel that function in cold temperature detection (Almaraz et al. 2014).

The relative expression of TRPM channels differs across the species under investigation. The zebrafish TRPM family consists of 11 members. There are two TRPM1 members, one TRPM2, one TRPM3, four TRPM4, one TRPM5, one TRPM6 and one TRPM7 member. TRPM8 is not expressed in zebrafish (Kastenhuber et al. 2013).

TRPM7

Among the TRPM eight members, TRPM7 is a fusion protein that consists of an ion channel linked to an alpha kinase domain, and therefore the protein is also known as “channel kinase”. TRPM7 is regulated by magnesium (Mg\(^{2+}\)) and Mg•ATP which were shown to inhibit channel activity (Nadler et al. 2001). In humans, TRPM7 (hTRPM7) allows the entry of Mg\(^{2+}\) into the cell. Mg\(^{2+}\) is involved in many different processes of the body. Some of the processes that utilize Mg\(^{2+}\) include regulation of ion channels such as hTRPM7, enzymatic reactions requiring adenosine triphosphate (ATP), cell proliferation and immunity (Pham et al. 2014).

TRPM7 was also shown to be a non-specific divalent ion channel that conducts trace metals and toxic metals. This suggests that due to TRPM7’s ability to conduct trace metals along with endogenous expression and constitutive activity, TRPM7 is a mechanism for influx of trace metals into the cells at the normal resting membrane potential. The divalent permeability of
TRPM7 was shown to consist of zinc and nickel as the two most permeable divalents followed by barium, cobalt, magnesium, manganese, strontium, cadmium and calcium (Monteilh-Zoller et al. 2003). TRPM7 has an outwardly rectifying current due to voltage-dependent block by magnesium and calcium (Kerschbaum et al. 2003; Nadler et al. 2001). A subunit of TRPM7 consists of six trans-membrane spans with a pore loop segment between span five and six that forms the pore and contains the ion selectivity filter (Li et al. 2007)(Fig. 2). Although TRPM7 is a non-specific divalent cation channel, it is also thought to be a primary mechanism for Mg$^{2+}$ homeostasis in the cell (Schmitz et al. 2003). Systemic magnesium levels are controlled by TRPM7. This was shown in a study that used mice heterozygously expressing wild-type TRPM7 and TRPM7 kinase deletion to show that these TRPM7 deficient mice developed hypomagnesemia due to insufficient magnesium absorption through the intestines (Ryazanova et al. 2010).

Magnesium (Mg$^{2+}$) is a common divalent cation in the human body. Mg$^{2+}$ deficiency in people represents a significant public health problem, as Mg$^{2+}$ regulates many cellular processes such as enzymatic reactions involving ATP, regulation of ion channels, such as TRPM7, as well as cellular proliferation, to name a few (Pham et al. 2014). Hypomagnesemia is defined as having a serum Mg$^{2+}$ level below 0.66 mmol/L with significant signs and symptoms that show up when serum Mg$^{2+}$ drops below 0.5 mmol/L. Some of the symptoms of hypomagnesemia found in humans can include, tremors, convulsions and even coma. Magnesium deficiency in rats has been linked to inflammation and insulin resistance, while increased extracellular magnesium reduces the inflammatory effects (Rayssiguier et al. 2010).
Magnesium also plays a significant role in cardiovascular health. A recent study performed a meta-analysis of previously published studies and found that the risk of cardiovascular disease events is reduced when dietary magnesium is increased. Increasing dietary magnesium intake from 150 mg per day to 400 mg per day showed the largest risk reduction for cardiovascular disease events (Qu et al. 2013).

TRPM7 has been implicated in many diseases. TRPM7 is thought to be involved in metastasis formation in breast cancer (Meng et al. 2013; Middelbeek et al. 2012), though the basis for this involvement is currently unknown. TRPM7 was shown to be involved with delayed neuronal death after brain ischemia by using small interfering RNA to knock down TRPM7, resulting in a reduction in delayed neuronal death after brain ischemia (Sun et al. 2009). TRPM7 is thought to be responsible for oxygen-glucose deprivation-induced neuronal cell death due to influx of Ca²⁺ and Mg²⁺ during oxygen glucose deprivation (Aarts et al. 2003; J. Zhang et al. 2011). TRPM7 is activated during oxygen glucose deprivation by the extracellular acidic conditions due to build up of carbon dioxide as well ATP depletion in the cell. Influx of Zn²⁺ due to TRPM7 activity has also been linked to neurotoxicity after brain ischemia (Inoue et al. 2010).

TRPM7 has been shown to play an important role in cardiac tissue. TRPM7 and TRPM6 like currents were shown to exist in pig and rat ventricular cardiac myocytes (Gwanyanya et al. 2004). TRPM7 was later shown to be functional in human atrial myocytes and TRPM7 expression levels are up-regulated in patients after atrial fibrillation (Zhang et al. 2012). TRPM7 calcium influx was also shown to be a key signal in human atrial fibroblast proliferation and differentiation. The human atrial fibroblasts from patients after atrial fibrillation also show up-regulation of TRPM7 (Du et al. 2010). These studies suggest the TRPM7 plays an important role.
in calcium entry in atrial fibroblasts. Blockage of TRPM7 may reduce cardiac fibrosis due to a reduction in calcium entry.

In addition to Mg$^{2+}$ and Mg•ATP sensitivity, TRPM7 is also sensitive to changes in osmolarity and pH. Increases in external osmolarity cause an inhibition in hTRPM7 outward current. Experimentally, the IC$_{50}$ for osmolarity has been shown to be 430 mOsm (Bessac and Fleig 2007). The pH affects the inward current of hTRPM7. As the pH drops below 7, the hTRPM7 inward current increases. The EC$_{50}$ for hTRPM7 current in regards to pH change was found to be 4.7 (Li et al. 2006). External pH changes can happen in the brain during cerebral ischemia (Mutch et al. 1984).

Past research has shown that hTRPM7 can be inhibited pharmacologically through the use of 2-aminoethoxydiphenyl borate (2-APB). 2-APB can be used for non-specific inhibition of hTRPM7 current. A dose-response has been performed on hTRPM7 to determine the concentration of 2-APB that inhibits hTRPM7 current by 50% (IC$_{50}$). The IC$_{50}$ for 2-APB on hTRPM7 current was shown to be 178 µm (Li et al. 2006). 2-APB is a non-specific inhibitor and has been shown to activate TRPM7’s sister channel kinase TRPM6 (hTRPM6). 2-APB potentiates hTRPM6 in a dose-dependent manner and was shown require 205 µM 2-APB to reach half maximal hTRPM6 current from baseline (EC$_{50}$) (Li et al. 2006).

TRPM6 is the sixth member of the TRPM family. TRPM6 is often referred to as the sister channel to TRPM7. The structure of TRPM6 is very similar to TRPM7. A subunit of TRPM6 consists of six trans-membrane spans with a pore loop segment between span five and six that forms the pore and contains the ion selectivity filter (Li et al. 2007; Chubanov et al. 2014). The divalent permeability of TRPM6 is similar to TRPM7 with a permeability profile of:
Zn > Ba > Mg > Ca > Mn > Sr > Cd > Ni. The major difference being that nickel is much less permeable than TRPM7 where nickel and zinc are the most permeable divalent ion (Li et al. 2006). TRPM6 subunits can form homomeric tetramers as well as heteromeric TRPM7/TRPM6 complexes that have their own unique properties (Chubanov et al. 2007; Z. Zhang et al. 2014). Similarly to TRPM7, TRPM6 contains a channel and alpha-kinase and as such is also known as a “chanzyme”. One of the major differences between TRPM7 and TRPM6 is that TRPM6 is not ubiquitously expressed. RT-PRC and Northern-blot analysis have shown that TRPM6 is located in intestines, kidney and testis (Schlingmann et al. 2002; Walder et al. 2002).

More recently a small molecule named Waixenicin A was discovered from an extract of soft coral that was shown to be a specific, potent, inhibitor of hTRPM7. This discovery led to a dose-response of Waixenicin A on hTRPM7 and determining that the IC₅₀ for Waixenicin A on hTRPM7 was 16 nM in the presence of physiological Mg²⁺ inside of the cell (Zierler et al. 2011). Waixenicin A offers a more reliable form of pharmacological inhibition than was previously available, without off-target effects.

Previously, attempts were made to develop a knock-out of mouse TRPM7 (mTRPM7) in order to assess the functional role of TRPM7, which led to the discovery that knocking out mTRPM7 is embryonically lethal at embryonic day 7.5 (Jin et al. 2008). This aspect of mTRPM7 limited the studies that could be performed in an attempt to characterize the channel kinase. There were, however, mutant forms of TRPM7 that were created to better understand the Mg²⁺ binding domains. A truncation of TRPM7 that removes the kinase domain after amino acid 1599 has been shown to lead to a non-functional TRPM7 channel (Matsushita et al. 2005). Another group investigated a truncated mutant of TRPM7 at amino acid 1569 and found that the resulting
truncated TRPM7 was partially functional (Schmitz et al. 2003). Most recently, a third group investigating truncated mutants of TPRM7 found that a truncation at amino acid 1510 resulted in a fully functional TRPM7 channel (Desai et al. 2012). These results suggest that between amino acid 1510 and 1599 in TRPM7 lies an area of interest for channel function.

Although mTRPM7 knockout in mouse is embryonically lethal to the mouse, there does exist a mutant TRPM7 in zebrafish that results in a non-functional TRPM7 channel that is not lethal to the zebrafish (Decker et al. 2014). Although the mutant zebrafish can survive without TRPM7, there are skeletogenesis and melanophore defects as well as the formation of kidney stones during their larval life. The mutant zebrafish also exhibits a touch unresponsiveness phenotype (Elizondo et al. 2005).

The ability of zebrafish to survive a loss of function mutation of TRPM7 makes zebrafish a desirable animal model to study the developmental effects of non-functional TRPM7. A recent study investigating the effects of loss of function TRPM7 on the development of zebrafish recently showed evidence that TRPM7 is involved in the function and maintenance of dopaminergic neurons (Decker et al. 2014). These findings are interesting, but the question remains how functionally similar zebrafish TRPM7 (drTRPM7) is to hTRPM7 and how relevant these results are to human disease and development.

Sequence analysis shows, drTRPM7 shares 72% sequence identity with hTRPM7 and 49% sequence identity with hTRPM6 (www.uniprot.org accessed 11/14/14). Different regions of interest were aligned within drTRPM7, hTRPM7 and hTRPM6 to determine if important regions in hTRPM7 and hTRPM6 are conserved in drTRPM7. The pore regions of hTRPM7 and hTRPM6 were aligned with drTRPM7 (Fig. 3) to determine similarities in pore sequences. The
pore region of drTRPM7, hTRPM7 and hTRPM6 are well conserved between the three channels (Li et al. 2007). The conserved “EVY” sequence within the pore domain has been suggested to determine divalent permeability in TRPM7 and contribute to the current voltage relationship as well as magnesium permeation block (Schnitzler et al. 2008). The conserved glutamate residue in the EVY sequence has also been shown to be involved in increased inward current during external low pH (Li et al. 2007).

The TRP domain is a highly conserved domain between all of the TRP channels, which was also conserved between drTRPM7, hTRPM7 and hTRPM6 (Venkatachalam et al. 2007). The coiled coil domain of each subunit is thought to be involved in homomeric and heteromeric complexes of four subunits of TRPM7 or TRPM6 or TRPM6/TRPM7 (Fujiwara et al. 2008). The coiled coil domain is conserved between drTRPM7; hTRPM7 and hTRPM6, suggesting that drTRPM7 may form heteromeric complexes or homomeric complexes with hTRPM7 and hTRPM6.

TRPM7 and TRPM6 both contain a kinase domain known as alpha kinase. The TRPM7 alpha kinase can phosphorylate serine and threonine residues in a magnesium dependent manner (Ryazanova et al. 2004). The TRPM7 alpha kinase has been shown to autophosphorylate itself as well as myelin basic protein and histone H3. Autophosphorylation does not appear to affect channel activity (Matsushita et al. 2005). There is also a magnesium nucleotide discrimination site within the kinase domain of TRPM7. Point mutations in the magnesium nucleotide discrimination render the TRPM7 channel unable to be regulated by magnesium nucleotides (Demeuse et al. 2006).
Also shown are two amino acid residues shown to be involved with hypomagnesemia with secondary hypocalcemia (HSH) in TRPM6. HSH is an autosomal recessive disorder that causes reduced serum magnesium, which is caused by reduced magnesium absorption in the intestines and increased magnesium excretion in the kidneys. This disorder has been linked to two mutations in TRPM6. The first mutation, found in patients with HSH, disrupts the formation of TRPM6/TRPM7 complexes, which suggest serine at position 141 in TRPM6 is critical for forming TRPM6/TRPM7 complexes (Chubanov et al. 2004). The second mutation identified in patients with HSH involves the proline located at position 1017 in the pore domain of TRPM6, which suggests that both TRPM7 and TRPM6 contribute to the pore domain in TRPM7/TRPM6 complex channels (Chubanov et al. 2007). Both of these mutations found in HSH patients are conserved within hTRPM7 as well as drTRPM7.

As of this writing, nothing is known about the biophysical characteristics of drTRPM7. It therefore remains to be explored whether zebrafish represents a good animal system to study human diseases linked to channel kinase deficiencies. In addition, differences in the biophysical characteristics of drTRPM7 as compared to hTRPM7 may have implications for the specific functional role of this channel in Mg\(^{2+}\) homeostasis in the zebrafish model.
<table>
<thead>
<tr>
<th>Human TRP7</th>
<th>Zebrafish TRP7</th>
<th>Human TRP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSQSWISTLRECVYIIPSSKDPHRCLPGQCICQQLVRCFCGLRVQ 50</td>
<td>HSQSWISTLRECVYIIPSSKDPHRCLPGQCICQQLVRCFCGLRVQ 50</td>
<td>MEQPVFRLQSKQWPKVFDRCSTLTPSSKNPHRCPVQLCGYCLRLGIGD 60</td>
</tr>
<tr>
<td>HACFTAGSLAMKSYDVKLGDHFNQAIENESVKEHETQSEPDAYGVINFQGSSHYSYRKYVR 110</td>
<td>HACFTAGSLAMKSYDVKLGDHFNQAIENESVKEHETQSEPDAYGVINFQGSSHYSYRKYVR 110</td>
<td>HAGIDSYSTISAA---------KGESEQWSESVEHTKSTPDFTINGQDGEHTHAKYIR 113</td>
</tr>
<tr>
<td>LSDDFVRALVIQLRMLWMLLMPLVIIIWSQGIGISEHQRKQLLWGGILRAVTTGAW 170</td>
<td>LSDDFVRALVIQLRMLWMLLMPLVIIIWSQGIGISEHQRKQLLWGGILRAVTTGAW 170</td>
<td>LSDDFVRALVIQLRMLWMLLMPLVIIIWSQGIGISEHQRKQLLWGGILRAVTTGAW 170</td>
</tr>
<tr>
<td>ILTGGVNTVAKVHGDLAESKCHRASSRKRCKTYGIAVPGWYKVEHNRDLGVRDVVAFYQOTLLN 230</td>
<td>ILTGGVNTVAKVHGDLAESKCHRASSRKRCKTYGIAVPGWYKVEHNRDLGVRDVVAFYQOTLLN 230</td>
<td>ILTGGVNTVAKVHGDLAESKCHRASSRKRCKTYGIAVPGWYKVEHNRDLGVRDVVAFYQOTLLN 227</td>
</tr>
<tr>
<td>PLSKLNVLNLHSHFILVDDGTGVGKYAEGVRLRERLEKTIQNOQHHARIHGGQGFVVALIF 290</td>
<td>PLSKLNVLNLHSHFILVDDGTGVGKYAEGVRLRERLEKTIQNOQHHARIHGGQGFVVALIF 290</td>
<td>PLSKLNVLNLHSHFILVDDGTGVGKYAEGVRLRERLEKTIQNOQHHARIHGGQGFVVALIF 287</td>
</tr>
<tr>
<td>EGGPNGVILTLELQYESPPVPVPCVCEGTGARAADLAYTHKQESEGNNLPQAAEDPDIIST 350</td>
<td>EGGPNGVILTLELQYESPPVPVPCVCEGTGARAADLAYTHKQESEGNNLPQAAEDPDIIST 350</td>
<td>EGPGPNGVILTLELQYESPPVPVPCVCEGTGARAADLAYTHKQESEGNNLPQAAEDPDIIST 347</td>
</tr>
<tr>
<td>KRTFNFGQNEALHLFQTLMCNEKRKELITVFHGSQDHQIDVAILTALLKGTNAPFDQ 410</td>
<td>KRTFNFGQNEALHLFQTLMCNEKRKELITVFHGSQDHQIDVAILTALLKGTNAPFDQ 410</td>
<td>KRTFNFGQNEALHLFQTLMCNEKRKELITVFHGSQDHQIDVAILTALLKGTNAPFDQ 407</td>
</tr>
<tr>
<td>LILTLDLWVRDIACRHFEVFYQQQQLVGLSLEQANLDAVARMDVFAPVFLKLIENGVMSMHKLT 470</td>
<td>LILTLDLWVRDIACRHFEVFYQQQQLVGLSLEQANLDAVARMDVFAPVFLKLIENGVMSMHKLT 470</td>
<td>LILTLDLWVRDIACRHFEVFYQQQQLVGLSLEQANLDAVARMDVFAPVFLKLIENGVMSMHKLT 467</td>
</tr>
<tr>
<td>TPELLELYNTQPQTPMTPLFHLVRDVQKQNLPQGYXITLIDILGVIYELMGMGTRCYTCTR 530</td>
<td>TPELLELYNTQPQTPMTPLFHLVRDVQKQNLPQGYXITLIDILGVIYELMGMGTRCYTCTR 530</td>
<td>TPELLELYNTQPQTPMTPLFHLVRDVQKQNLPQGYXITLIDILGVIYELMGMGTRCYTCTR 527</td>
</tr>
<tr>
<td>KRFRIYLNGLGODRSGRNTSSSTTPQFLRXKHFHERNADERKEKMRNHFHKFTREHYK 590</td>
<td>KRFRIYLNGLGODRSGRNTSSSTTPQFLRXKHFHERNADERKEKMRNHFHKFTREHYK 590</td>
<td>KRFRIYLNGLGODRSGRNTSSSTTPQFLRXKHFHERNADERKEKMRNHFHKFTREHYK 582</td>
</tr>
<tr>
<td>IDTVEHEGKKKTRIKKIENDDIDPETKFPYPLNEIJLIWACLKMQVNFRLWQGEGESA 650</td>
<td>IDTVEHEGKKKTRIKKIENDDIDPETKFPYPLNEIJLIWACLKMQVNFRLWQGEGESA 650</td>
<td>IDTVEHEGKKKTRIKKIENDDIDPETKFPYPLNEIJLIWACLKMQVNFRLWQGEGESA 641</td>
</tr>
<tr>
<td>KALVACIYRSMAYEKOSLVDVDSKELEKQYSNDFGWALVELEGSFRODENTNAWMLLT 710</td>
<td>KALVACIYRSMAYEKOSLVDVDSKELEKQYSNDFGWALVELEGSFRODENTNAWMLLT 710</td>
<td>KALVACIYRSMAYEKOSLVDVDSKELEKQYSNDFGWALVELEGSFRODENTNAWMLLT 701</td>
</tr>
<tr>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 770</td>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 770</td>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 761</td>
</tr>
<tr>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 770</td>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 770</td>
<td>YEKLKNWNSNSCTCLAVSSLRFLRPVFVHTQCTOQLSLDDKMKNLHNRNSWYKVILSILVPA 761</td>
</tr>
<tr>
<td>Species</td>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>ILLLEYTKAEMSHIPQSDAQHMTDMDSDENFPQHTITEPEKVFKEVRLIDSNFGKNE 829</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>ILLLEYTKAEMSHIPQSDAQHMS-EDSHEHLQ-HPPDIQMDVKFKEVRS- DANEASKE 818</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>ILLTEFKSCEAEMSHVPSQDPFQWNYDSQANNA-SKSSERASVKEYDERLGEDKLDNEQH 815</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>MEIQKMSKSSLPTITRFKAFYAHIPKVFVNFNTLALYGLFMLYTFYTVLVQMLQFSPQSVLYW 889</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>LEVLSVRSRKLFLPKHPIFVVFVNTLYTFFYLQFVVMKKLQDSFQPSQWLV 878</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>FLESGSHQHPWTRIRVYFEFAPVKIFVMTYALFAMFLYTFYVLMQVMPQPSQVLYW 875</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>IAYFTTAIEKVFIEF MPSAEKGVQKIDFVFDYFISDTIAISFIIFGFLRFAGKWFNI  949</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>ILYFTTAVEKIRFMEMVESMGKSGKIQIKIDFVFDYFISDTIAISFIIFGFLRFAGKWFNI  949</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>SIYFTNAIEVICIEPSKFGTVKQVWISLWYNLTETAVIFLSFGVLMRWDG-----  931</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>ANAYDNHFQVAOLYCMINIFWYVRLDLFLAVNQACAGPYMVMGKMWANMYVIVNIAL  1009</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>-----SEVFIAIRVCMINIFWYVRLDLFLAVNQACAGPYMVMGKMWANMYVIVNIAL  988</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>-----PPFIITAGRLIYCIIDIFIFSFVRSLDIFVAFHVGMQYPVMTIAKTVANKYNIVIANNI  986</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>VLLSGFVPRKAILYFHEAPSWTLAKDIVFHFIPWNIFG_VYAELIVCANDS-----VIFPQICG 1067</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>VLLSGFVPRKAILYFHEAPSWTLAKDIVFHFIPWNIFG_VYAELIVCANDSSEEHVRDCT 1048</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>VLLSGFVAKIILFSKPSWTSLARDIVFHPYWMTG_VYAGEIVCQSSQ-------PCS  1040</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>PGTWLFPLQLFQYLVYIMMVILIAIFPNNVYLVQKASINLVQKYFHEFMAYHEKPV  1127</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>TGVVTLFPLQLFQYLVYIMMVILIAIFPNNVYLVQKASINLVQKYFHEFMAYHEKPV  1127</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>PGFSFLFLQLQYLVYIMMVILIAIFPNNVYLVQKASINLVQKYFHEFMAYHEKPV  1108</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>LPPPLILISIVSFLCCICKRKR-KDKTDGFKFLFTEDEQKHLFDEEEQQCVEMFNE  1184</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>LPPPLILISIVSFLCCICKRKR-KDKTDGFKFLFTEDEQKHLFDEEEQQCVEMFNE  1184</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>LPPPLILISIVSFLCCICKRKR-KDKTDGFKFLFTEDEQKHLFDEEEQQCVEMFNE  1184</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>KDDKFHGSGSEIRLVTREAVQNCIQKVEQVDRNYKIQLSGLQIQLHGQIQLDQALTVD  1244</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>KDDQFHQSGSEIRLVTREAVQNCIQKVEQVDRNYKIQLSGLQIQLHGQIQLDQALTVD  1244</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>KDDQFHQSGSEIRLVTREAVQNCIQKVEQVDRNYKIQLSGLQIQLHGQIQLDQALTVD  1244</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>TLKTILAQKASEAVK------HNE------------ITRELSISKHLAQN------LIDD  1281</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>TLKTILAQKASEAVK------HNE------------ITRELSISKHLAQN------LIDD  1281</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>TLKTILAQKASEAVK------HNE------------ITRELSISKHLAQN------LIDD  1281</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>GPTVQSCWVKKHGTVLSSSLPQDGSLENN-PFHCMNLMKDKDIDPOCN  1329</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>GPTVQSCWVKKHGTVLSSSLPQDGSLENN-PFHCMNLMKDKDIDPOCN  1329</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>GPTVQSCWVKKHGTVLSSSLPQDGSLENN-PFHCMNLMKDKDIDPOCN  1329</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>FGQDLP-------AVPFKREFNFPEAGSSGALPFSAVS--------PPELRO  1367</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>FGQDLP-------AVPFKREFNFPEAGSSGALPFSAVS--------PPELRO  1367</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>FGQDLP-------AVPFKREFNFPEAGSSGALPFSAVS--------PPELRO  1367</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>______-KLAVGELLKIFKNNOKLGLSSSSTSHLSSFPPTKVFVSTES  1407</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>______-KLAVGELLKIFKNNOKLGLSSSSTSHLSSFPPTKVFVSTES  1407</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>______-KLAVGELLKIFKNNOKLGLSSSSTSHLSSFPPTKVFVSTES  1407</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>DEPSKHEPAILLDGDQDK-AEQLVPLCTEPEMTMSFLSQAKMTQGGYFVWAF  1457</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>DEPSKHEPAILLDGDQDK-AEQLVPLCTEPEMTMSFLSQAKMTQGGYFVWAF  1457</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>DEPSKHEPAILLDGDQDK-AEQLVPLCTEPEMTMSFLSQAKMTQGGYFVWAF  1457</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>QPSCKSHLETGKDQETVCSC-----------KATEGDNTEFGA  1439</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>QPSCKSHLETGKDQETVCSC-----------KATEGDNTEFGA  1439</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>QPSCKSHLETGKDQETVCSC-----------KATEGDNTEFGA  1439</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>SDEGTETGYVSIRKKQWCLPTSDCDSDSRESQHKQAQQDSLSDNSDTRASQESCEVP 1517</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>SDEGTETGYVSIRKKQWCLPTSDCDSDSRESQHKQAQQDSLSDNSDTRASQESCEVP 1517</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>SDEGTETGYVSIRKKQWCLPTSDCDSDSRESQHKQAQQDSLSDNSDTRASQESCEVP 1517</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>FVGHRSMD------LQRFKETSINKIKLINSNNNTSENTLKRVSILAGF  1481</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>FVGHDOFPE------GQRSIPKESAVSSRKRTRDVMNRTVS1AGF  1395</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>FVGHRSMD------LQRFKETSINKIKLINSNNNTSENTLKRVSILAGF  1481</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>WQFQNTFSWPNILRYPRFARFHPFRHFHHEKIMKCIKKNLSGSSETGGAWVKAMLT 1577</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>WQFQNTFSWPNILRYPRFARFHPFRHFHHEKIMKCIKKNLSGSSETGGAWVKAMLT 1577</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>WQFQNTFSWPNILRYPRFARFHPFRHFHHEKIMKCIKKNLSGSSETGGAWVKAMLT 1577</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>---TDCRTSIFVHSKQAE-KISR------RP5  1504</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>---TDCRTSIFVHSKQAE-KISR------RP5  1504</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>---TDCRTSIFVHSKQAE-KISR------RP5  1504</td>
<td></td>
</tr>
<tr>
<td>Human TRPM7</td>
<td>KDRKLSSKXNYQSLQPIITVACQSD-QLNFEPGENISSEEESKNWFTVSFSHTG 1636</td>
<td></td>
</tr>
<tr>
<td>Zebrafish TRPM7</td>
<td>KDRKLSSKXNYQSLQPIITVACQSD-QLNFEPGENISSEEESKNWFTVSFSHTG 1636</td>
<td></td>
</tr>
<tr>
<td>Human TRPM6</td>
<td>KDRKLSSKXNYQSLQPIITVACQSD-QLNFEPGENISSEEESKNWFTVSFSHTG 1636</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3: human TRPM7, human TRPM6 and zebrafish TRPM7 alignment

An alignment performed on Human TRPM7 (top row), Zebrafish TRPM7 (middle row) and Human TRPM6 (bottom row). Regions of interest are highlighted with a color or the font color has been altered for ease of identification. The TRP domain is highlighted in purple, Coiled coil domain is blue font, Mg\*NTP discrimination site highlighted in red, divalent cation filter highlighted in blue, Pore region highlighted in green, Kinase domain highlighted in yellow, Mutation in human TRPM6 leading to hypomagnesemia with secondary hypocalcemia have red font, Acid sensing amino acid is highlighted in blue with yellow font and the location of truncated mutants of drTRPM7 have blue font.
Hypothesis

Human TRPM7 (hTRPM7) and mouse TRPM7 nucleotide sequence share 94.3% sequence identity (www.uniprot.org accessed 3/30/15). Experimentally deleting the TRPM7 protein is embryonically lethal at day 7.5 in mouse (Jin et al. 2008). In comparison, drTRPM7 shares 72% sequence identity with hTRPM7 (www.uniprot.org accessed 11/14/14) and a loss of function mutation of drTRPM7 is not embryonically lethal. Furthermore, removal of the TRPM7 kinase domain leads to strongly reduced channel activity, whereas cleaving the protein’s C-terminus shortly after the 6th transmembrane domain recovers channel activity. The underlying mechanism for this observation is unknown, as are the biophysical characteristics of drTRPM7.

Based on these observations, I hypothesize that

1. The biophysical characteristics of drTRPM7 are identical to hTRPM7 and
2. The site that coordinates binding of Magnesium to the TRPM7 protein is located between trans-membrane segment 6 and the kinase domain.

I tested these hypotheses via the following Specific Aims:

Specific Aim 1: Investigation of the biophysical characteristics of wild-type drTRPM7.

To this end, a HEK293 T-REx cell line will be established that heterologously overexpresses the drTRPM7 protein upon tetracycline-induction. This will facilitate whole-cell patch-clamp experiments to determine the biophysical characteristics of drTRPM7 currents, including regulation by intracellular Mg^{2+} and Mg•ATP, the channel’s divalent ion permeability, inhibition by 2-Aminoethoxydiphenyl borate (2-APB) and a specific inhibitor, Waixenicin A. Furthermore, drTRPM7 will be tested against sensitivity to low pH and high osmolarity.
**Specific Aim 2: Identification of a magnesium-regulatory protein domain.**

We have obtained pcDNA containing either drTRPM7 wild-type or one of three truncated drTRPM7 mutant genes (see Figure 1). The drTRPM7 mutant names are Delta Kinase (DK, truncating drTRPM7 at amino acid (aa) 1478), Coiled Coil Region (CCR, truncated at aa1256), and No CCR, truncated at aa 1122. These truncated mutants will be stably overexpressed in the HEK293 T-REx cell line followed by whole-cell patch clamp experiments in order to investigate whether these regions play a role in regulating/coordinating Mg\(^{2+}\) and Mg•ATP binding on drTPRM7.

**Specific Aim 3: Determination whether heterologous drTRPM7 reflects the biophysical properties of native drTRPM7.**

Since TRPM7 is expressed ubiquitously in every cell type and tissue, the zebrafish liver cell line ZFL can be used to study natively expressed drTRPM7. Key biophysical properties of drTRPM7 will be assessed and compared to the data obtained in Specific Aim 1. Specifically, native drTRPM7 will be probed for its Mg\(^{2+}\) sensitivity and pharmacological profile using 2-APB.
Chapter 2: Model Systems and Methodology

Model systems

*Human embryonic kidney cells (HEK293)*

Two model systems were selected for this project. The first model system selected was heterologous human cell expression. Heterologous human cell expression offers many benefits to study the biophysical characteristics of drTRPM7. The heterologous expression in HEK293 T-REx cells overexpressed drTRPM7, which offered large robust currents to record with a more favorable signal to noise ratio than the endogenous expression of drTRPM7. The large robust currents were much larger than the endogenous hTRPM7 current, which helped minimize the current contamination from endogenous hTRPM7.

*Zebrafish liver cells (ZFL)*

The second model system is an endogenous expression system for drTRPM7 in Zebrafish liver cells (ZFL). The ZFL cells were ideal for studying drTRPM7 in a native context. The ZFL cells do not express human TRPM7, which is a benefit over the HEK293 cells. Studies in human tissue showed that TRPM6 expression is located primarily in the intestine and kidney, not in the liver (Schlingmann et al. 2002). It was presumed that TRPM6 was not expressed in ZFL cells due to previous studies reporting TRPM6 expression in the intestine and kidney. The ZFL cells also offer the opportunity to study cellular regulation, such as magnesium homeostasis as well as ATP depletion.
Molecular Biology

Transformation of bacteria

The transformation of bacteria was used to create more pcDNA that can later be used for experimentation. The pcDNA that was transformed for amplification was a pcDNA 5TO plasmid (Invitrogen Cat. No. V1033-20) containing either drTRPM7 wild-type, drTRPM7 DK, CCR, or No CCR truncation mutants (Fig. 4). These plasmids were used to create stably expressing HEK293 T-REX cell lines that will overexpress drTRPM7 upon exposure of cells to tetracycline. The process of pcDNA amplification was achieved through the use of competent bacteria cells known as DH5 alpha cells (Invitrogen Cat. No. 18258-012). The vial of competent DH5 alpha cells was thawed on ice and the pcDNA was added to the competent cells. The competent DH5 alpha cells and pcDNA mixture was incubated on ice for 30 minutes. After the 30 minute incubation, the competent DH5 alpha cells and pcDNA mixture was heat shocked for 45 seconds at 42°C. After the heat shock the DH5 alpha cell mixture was returned to the ice for incubation for the next two minutes. Room temperature S.O.C. media (Invitrogen Cat. No. 15544-034) was added to the DH5 alpha mixture and incubated in a 225 rpm shaker at 37°C for one hour. After the incubation, 100 μL and 400 μL of cells were plated on agar plates and grown overnight at 37°C. The next day colonies were harvested and grown in 200 mL LB broth media at 37°C overnight in preparation for Maxi prep DNA purification.
Figure 4: The 5TO and 6/TR pcDNA plasmids

6/TR plasmid contains the tet repressor protein (gray), which can bind the tet operator sequence (purple) on the 5TO pcDNA plasmid to regulate expression of the gene of interest. 5TO plasmid contains 2 tet operator sequences that allows the tet repressor protein to bind and allows for inducible expression of drTRPM7. In the presence of tetracycline (orange) the tetracycline repressor is not bound to the tet operator sequence which allows for transcription of drTRPM7.
DNA purification

The 200 mL LB broth cultures contained bacteria that harbor the pcDNA of interest. The DNA purification step granted the ability to separate the bacteria and pcDNA. Standard protocols were used to purify the DNA from the transformed DH5 Alpha cells. The DNA purification kit used was Qiagen Plasmid Maxi Kit (Qiagen Cat. No. 12162) the manufacturers provided protocol was used for DNA purification. The Qiagen Plasmid Maxi Kit was used to purify cultures grown in 200 mL LB broth to produce purified plasmid DNA. The purified plasmid DNA was then linearized via a restriction enzyme digest.

Linearization of 5TO

A restriction enzyme that cuts a specific nucleotide sequence was used to linearize the pcDNA 5TO plasmids containing drTRPM7 wild-type and truncated mutants (DK, CCR and No CCR). The restriction enzyme made a single cut in the 5TO plasmid backbone. The restriction enzyme selected was sspI (New England Biolabs Cat. No. R3123L) for linearization due to sspI cutting sequence 5' AATATT 3', which is a sequence that only occurs once in the pcDNA 5TO plasmid containing all four versions of drTRPM7. The Restriction enzyme reaction contained 1 µL sspI enzyme, 5 µg 5TO plasmid containing drTRPM7 or truncations, 1.5 µL NEB buffer and 7.5 µL dH₂O for a total reaction of 15 µL incubated for 1 hour at 37 °C. After the incubation period the reaction was heat inactivated at 65 °C for 20 minutes. The 5TO plasmid was visualized in an agarose gel to verify that the pcDNA 5TO plasmid was linearized.
DNA gel electrophoresis

DNA gel electrophoresis was performed after DNA restriction enzyme digest with sspI restriction enzyme to check for DNA linearization. The DNA gel electrophoresis was performed using 1% agarose gel in TAE buffer. The TAE buffer contained 40 mM Tris, 20 mM glacial acetic acid and 2 mM EDTA at a pH of 8.3. The restriction enzyme and pcDNA product were mixed with 10x loading buffer to a final concentration of 1x. 1 µg of digested pcDNA was run through the 1% agarose gel at 100 V for ~ 1 hour. The gel was visualized using the SYBR safe DNA gel stain (Invitrogen Cat. No. S33102)(Fig. 5).

Figure 5: Linearization of drTRPM7 plasmid

Uncut and linearized 5TO plasmid containing drTRPM7 and drTRPM7 DK truncation. The uncut plasmid migrated quicker through the agarose gel than the sspI digested linearized plasmid due to the uncut plasmid being supercoiled. The uncut lanes also contain a small band above the supercoiled that may be a small amount of nicked plasmid.
Cell Culture Methods

Maintenance of cell culture:

The HEK293 Wild-type cells were maintained in DMEM (Corning Cat. No. 10-014-CV) supplemented with 10% fetal bovine serum (Invitrogen Cat. No. 16140071) and the appropriate selective antibiotics, if required; 200 µg/mL Hygromycin B (Invitrogen Cat. No. 10687-010) and 5 µg/mL Blasticidin (Invivogen Cat. No. ant-bl-1). The HEK293 cells were incubated at 37 °C in 5% CO₂. The ZFL zebrafish cells were maintained in media that contained 50% L-15 (ATCC Cat. No. 30-2008), 35% DMEM HG (GIBCO Cat. No. 12100), 15% Ham’s F12 (GIBCO Cat. No. 21700), 0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL bovine insulin, 50 ng/mL mouse EGF, 5% heat-inactivated fetal bovine serum and 0.5% trout serum. The ZFL cells were incubated at 28 °C without added CO₂.

Production of inducible stable expression cell line:

The HEK293 T-REx cell line with the 5TO plasmid provided a system that enabled the inducible overexpression of a particular protein of interest, in this case drTRPM7 Wild-type and the drTRPM7 truncated mutants DK, CCR and No CCR (provided by Dr. Andrew Scharenberg, University of Washington Seattle Children’s Research Institute, see Fig. 1). Stable inducible overexpression cell lines were developed with T-REx system (Invitrogen Cat. No. R710-07). The T-REx system is a tetracycline inducible overexpression system that contains the TetR gene that encodes for a tetracycline repressor under control of the cytomegalovirus (CMV) promoter and an antibiotic resistance element (Fig. 4). The tetracycline repressor is one part of the inducible expression. The second part of the inducible expression is the two-tetracycline operator sequence located on the 5TO plasmid that the tetracycline repressor can bind to give control of expression.
of the drTRPM7 wild-type or one of the three truncated mutants. Addition of tetracycline caused a binding between tetracycline and the tetracycline repressor protein. The tetracycline binding to the tetracycline repressor caused a conformational change in the tetracycline repressor protein and released it from the operator sequences, which allowed transcription initiation.

Stable inducible overexpressing HEK293 T-REx cell lines were generated by growing HEK293 T-REx cells in 10 cm round dishes to ~80% confluency. After confluency was reached in the HEK293 T-REx cells, the media was aspirated and replaced with 5 mL optimem (Invitrogen Cat. No. 31985070), 21 µL lipofectamine 2000 (Invitrogen Cat. No. 11668027) and 15 µg pcDNA 5TO plasmid (Invitrogen Cat. No. V1033-20), which contained drTRPM7 wild-type or one of the three drTRPM7 truncated mutants, and incubated at 37 °C and supplemented with 5% CO₂ overnight. After 24 hours the media was aspirated and replaced with fresh DMEM and 10% FBS containing media and left to incubate at 37 °C supplemented with 5% CO₂ for another 24 hours. After 24 hours with antibiotic free media, the media was aspirated and replaced with DMEM, 10% FBS, 200 µg/mL Hygromycin (5/TO) and 5 µg/mL Blasticidin (6/TR) to select for stable transfectants. The media was aspirated and replaced every three days until colonies of cells formed on the 10 cm dish. The colonies of cells were harvested by utilizing cloning cylinders (Corning Cat. No. 3166-8). The manufacture’s protocol was followed for the use of cloning cylinders. The colonies that were harvest were transferred to 12-well plates with the appropriate antibiotic selection media for growth. After standard passaging in the 12-well plates and t-25 flasks, each colony was seeded into two wells in a 12-well plate. One well was left uninduced and the other well was induced with tetracycline. Immunoblot analysis was performed to verify expression of drTRPM7 wild-type and the three truncated mutants.
**Protein biochemistry**

*Antibodies*

The primary antibody that was used for the western blot technique was anti human influenza hemagglutinin (anti-HA tag antibody) (Roche Cat. No. 11 867 423 001), which was used at a concentration of 100 ng/mL. This anti-HA tag antibody recognizes a short peptide sequence that was added to all four of the drTRPM7 channels at the beginning of the nucleotide sequence. The secondary antibody used was Anti-rat horseradish conjugated secondary antibody peroxidase at a dilution of 1:80000.

The loading control was performed by stripping the blot and probing for GAPDH. This was achieved through the use of GAPDH antibody (abcam Cat. No. ab8245) at a 1:5000 dilution. The secondary antibody used was Anti-mouse horseradish conjugated secondary antibody (GE Healthcare Cat. No. NA931) at a dilution of 1:12500. The loading control showed that the samples were loaded equally into each well.

*Cellular preparation for Western Blot*

The cells were prepared for a western blot to confirm expression of drTRPM7. Sixteen hours prior to cell collection 1 µg/mL tetracycline was added to the media to induce expression of drTRPM7 wild-type or the three truncated mutants of drTRPM7. The cells were collected by pipetting the media in the 75-cm² flask over the cells to detach the cells from the flask. The media that contained the cells was then transferred to a centrifuge tube to be centrifuged for five minutes at 1000 rpm to form a pellet of HEK293 cells. The media was aspirated and the cells were washed with 1.5 mL of cold phosphate buffered saline (PBS). After the PBS wash the cells
were transferred to a microcentrifuge tube and centrifuged for five minutes at 12,000 rpm at 4 °C and then placed on ice. The supernatant was aspirated and the cells were lysed with 0.5 mL lysis buffer that consisted of: 10 mM Tris-HCl, 75 mM NaCl, 5% glycerol, 0.5% triton, 5mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease cocktail. After the lysis buffer was added the cells were incubated for 30 minutes at 4 °C. After incubation the cells were centrifuged at 12,000 rpm for 5 minutes at 4 °C and the lysate was transferred to a fresh microcentrifuge tube. 40 µg of protein was combined with NuPAGE LDS sample buffer and NuPAGE Reducing Agent with deionized water to final volume of 20 µl. The samples were heated to 95 °C for 8 minutes.

**Western Blot**

The boiled samples were loaded into a NuPAGE 4% - 12% gel (Invitrogen Cat. No. NP0322BOX) and ran at 200 V for 1 hour. A polyvinyl difluoride (PVDF) membrane was prepared and activated with methanol. The gel was then transferred to the PVDF membrane overnight at 15 V at 4 °C in a transfer buffer consisting of 25 mM Tris base and 0.192 M Glycine. The next morning, after the transfer took place, the PVDF membrane was blocked 5% (w/v) dry non-fat milk solution at room temperature. The primary antibody was dissolved in primary antibody diluent which contained: 0.5% dry non-fat milk, 15 mM Tris base, 150 mM NaCl, 0.05% (v/v) Tween-20 and 0.05% NaN₃ and incubated with the membrane for approximately 2 hours at 4°C with gentle shaking. After incubation, the membrane was then washed 3 times with TBS Tween which contained: 15 mM Tris base and 150 mM NaCl, for 5 minutes at room temperature with gentle shaking. New TBS Tween was added for each wash. The membrane was then incubated with the appropriate secondary antibody in TBS Tween with
0.5% (w/v) dry non-fat milk solution for 1 hour at 4 °C. After the secondary antibody incubation the PVDF membrane was washed 3 more times with TBS Tween and then the PVDF membrane was exposed to ECL solution (Life Technologies Cat. No. 34080) for 4 minutes. The ECL solution was drained off and the membrane was exposed to film and the film was developed (Fig. 6).

The western blot showed that when the transfected cells were induced with tetracycline, the HA-tag could be detected. The western blot also showed that when the cells were uninduced or wild type HEK293 were used, there was no detection of the HA-tag. This result suggests that the HA-tagged drTRPM7 expression was dependent on tetracycline being present in the media. The overexpression was confirmed through whole cell patch clamp and found that the drTRPM7 plus endogenous hTRPM7 in the HEK293 cells was about ten times higher than endogenous hTRPM7 in HEK293 cells.
Figure 6: Immuno blot detection for HA-tag drTRPM7

A western blot showing resolved extracts from Wild-type HEK293 cells and several colonies of drTRPM7 inducible overexpression HEK293 cells that are induced (I) or uninduced (U) that have been probed for the HA-tag. The blot used to detect the HA-tag was then reprobed for the GAPDH protein.
Electrophysiology

Patch-Clamp Technique

The development of the patch-clamp technique offered the opportunity to examine the characteristics of specific ion channels (Neher et al. 1976). The patch-clamp technique is generally recognized as clamping the voltage across the plasma membrane to a certain value to measure the resulting flow of ions through either single ion channels, or ion channels in the membrane of the whole cell. The patch-clamp technique is also used to control the amount of current and measure the voltage changes across the membrane. The patch-clamp is made possible through the use of glass micropipettes that contain a recording electrode that is tightly sealed to the plasma membrane of the cell to be recorded. The discovery of the tight seal through the formation of a “gigaseal” allowed for more precise recordings that contained less background noise (Sigworth et al. 1980). The resistance can be monitored to determine when a gigaseal has formed; a gigaseal is formed when the resistance reaches $10^9 \Omega$.

Various recording configurations have been described in the literature: cell-attached, inside-out, outside-out, whole-cell and perforated patch (Hamill et al. 1981; Ashcroft et al. 2013). Once the gigaseal has formed, the cell attached recording method can be utilized. The cell-attached method can be used to record current from a single ion channel. The inside-out method is achieved when removing the pipette from the cell during cell-attached configuration. While using the inside-out method of recording the external solution the cells are bathed in is used for the cytoplasm side of the ion channel while the solution in the pipette models the solution outside of the cell in inside-out configuration. The inside-out method of patch clamping
allows for full access to the solutions inside and outside of the cell to record as few as a single ion channel.

The whole-cell method of patch-clamp recording can be used to measure all of the ion channels in the cell’s plasma membrane. Being in cell-attached configuration and using a short burst of suction to rupture the plasma membrane and create a hole in the plasma membrane achieve this method. The hole in the membrane allows for solution inside the pipette to perfuse into the cell and the external solution the cells are bathed in to model the solution outside of the cell. The whole-cell method allows for recording the average response of all of the ion channels in the plasma membrane. The whole-cell method can also be used to detect exocytosis in secretory cells by monitoring the cells capacitance.
Figure 7: Patch Clamp Configurations:

A) The glass pipette tightly sealed to the membrane (gigaseal) in cell-attached configuration. B) Inside-out configuration where a small part of the membrane is removed with the inside of the membrane exposed to the external solution. C) Outside-out configuration where a small part of the membrane is removed with the outside of the cell membrane exposed the external solution. D) Whole-cell configuration, which is used to measure the total current of the whole cell. E) The perforated patch configuration utilizes pore forming antibiotics to gain electrical access to the cell’s interior (Ashcroft et al. 2013)
Patch Pipettes

The glass pipette resistances used for recording HEK293 T-REx cells was between 2.5 - 3.5 MΩ when filled with internal solution. The ZFL zebrafish cells required a smaller tip to form a tight seal; a resistance of 4 MΩ when the pipette was filled with internal solution was selected for the ZFL cells.

Whole Cell Patch-Clamp

To measure the small currents, two electrodes need to be present. The first electrode was a borosilicate glass recording electrode that contained a chloride-coated silver wire immersed in a salt solution modeling cytosolic salt concentrations. The tip of this glass electrode was in contact with the plasma membrane. The glass electrode was created with a Sutter p-1000 micropipette puller. The glass electrode that was pulled using the Sutter p-1000 micropipette puller was pulled to have a resistance of 2.5 MΩ when filled with internal salt solution. The ZFL cells required higher resistant pipettes to form a gigaseal, the ZFL glass pipette resistance was 4 MΩ. The tip was fire polished to assist in the formation of a tight giga-seal on the plasma membrane prior to rupturing the patch of membrane under the pipette tip, which allowed access to the cell’s interior. The second electrode served as reference electrode that was placed in the external solution the cells were bathed in. Both of the electrodes were chloride-coated silver wires. The glass electrode injected sufficient current into the cell to clamp the voltage of the plasma membrane to the desired level. The amount of current needed to clamp the voltage of the plasma membrane represented the inverted actual flow of ions through ion channels as well as any leakage currents through space between the glass pipette and the plasma membrane. The tiny current signal (in the order of pico Ampere (pA) or nano Ampere (nA)) measured by the glass
electrode was 10X amplified by a pre-amplifier connected to the chloride wire in the glass pipette. The pre-amplifier used for the current project was part of the EPC9 Patch Clamp Amplifier system (HEKA, Germany). The EPC9 amplifier digitalized the amplified analog signal before it was sent to the computer interface for digital recording, in this case a Macintosh PowerPC G4 (Apple, USA). Patchmaster (HEKA, Germany) was the software used to control current injection and storage of acquired data.

The patch clamp experiments for drTRPM7 were designed to have a holding potential of 0 mV while using a voltage ramp from -100 mV to +100 mV over 50 ms at 0.5 Hz. The currents reported in the results section were obtained from the current sizes measured at -80 mV and +80 mV. These potentials were selected based on -80 mV being a physiological potential and +80 mV was selected to be symmetrical with the physiological potential of -80 mV. A liquid junction correction of 10 mV was used. The electrophysiology and statistical analysis was performed on Fitmaster (version 2.69; HEKA, Germany) and Igor software (version 6.34; WaveMetrics, USA).

For patch-clamp experiments, cells were plated on glass coverslips. Before experimental use, the coverslips were placed in to a patch chamber and secured with vacuum grease. The cells were bathed in external solution that contained the sodium ringer (see solution section). The cells were visualized through a microscope (Axiovert 35, Zeiss) and camera that displayed to a monitor. An Eppendorf micromanipulator was utilized to adjust the stage. The glass electrode was filled with internal solution (see solution section) and mounted on a pipette holder with the chloride-coated sliver wire and attached to the pre-amplifier. Tubing was attached to the pipette holder to allow for negative or positive pressure manipulation of the solution in the glass electrode. A cell was then selected and the glass pipette was maneuvered to the cell surface and
suction was applied to the cell through the glass. Suction was used to increase the seal between glass electrode tip and cell membrane. The quality of the seal was monitored through the reduction of leak currents between glass electrode and plasma membrane and measured as an increase in seal resistance. A seal resistance of 1 GΩ indicated a seal tight enough to initiate the rupture of the membrane under the glass electrode tip to gain physical access to the inside of the cell. The internal solution was perfused into the cell and additionally the voltage across the plasma membrane was now set to the desired voltage, which started the whole-cell patch-clamp experiment.

**Solutions**

The standard external solution used contained (in mM) 140 NaCl, 2.8 KCl, 2 MgCl₂, 10 Glucose, 10 HEPES, and 1 CaCl. The divalent profile used a modified external solution that contained: 140 NMDG, 10 glucose, 10 HEPES and 10 CaCl₂. The divalent profile also used a modified application solutions that contained: 140 NMDG, 10 glucose, 10 HEPES and 10 of the divalent studied (MgCl₂, NiCl₂ or ZnCl₂ etc.). The sodium in the standard external solution was replaced with NMDG, a TRPM7 impermeable molecule, to ensure all inward current was due to divalent ions flowing into the cell as TRPM7 is permeable to sodium in divalent free conditions. The standard internal solution used contained 140 Cs-Glutamate, 8 NaCl, 10 HEPES, and 10 Cs-BAPTA. The solutions had their pH adjusted to 7.2 with either NaOH or CsOH respectively. The osmolarity ranged from 290 mOsm – 330 mOsm. The solutions used for pH dose-response below 6.0 were, 140 NaCl, 2.8 KCl, 2 MgCl₂, 10 Glucose, 10 MES, and 1 CaCl and pH was adjusted to appropriate pH with NaOH. The MES buffer was selected for low pH external solutions over HEPES due to MES having a lower buffering capacity. Mg-ATP dose-responses
internal solution 120 Cs-Glutamate, 8 NaCl, 10 HEPES, 10 Cs-BAPTA was used. Free ions were calculated using WebmaxC Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm).

Analysis

The statistical analysis was performed on Fitmaster (version 2.69; HEKA, Germany) and Igor software (version 6.34; WaveMetrics, USA). Data were normalized to cell size as current density (pA/pF) or normalized to the time point right before application (I/I\{\text{time}\}) and displayed as mean ± standard error of mean (s.e.m.). The dose-response curves were generated by the fit function f(x) = ([Maximum current] * (1/(1+(Estimated IC\text{50} / x)^{\text{Hill coefficient}}))). The IC\text{50} or EC\text{50} values calculated with the dose-response curve function were reported with ± standard deviation. The p-values were calculated using Microsoft Excel version 14.1.2 using the two-tailed paired Student’s T-test.
Chapter 3: Biophysical Characterization of drTRPM7

1. Introduction

TRPM7 is a key component of magnesium homeostasis in the cell and has been implicated in many diseases. A better understanding of TRPM7 may provide crucial information about diseases and pathologies that have been linked to TRPM7. The channel kinase hTRPM7 has been biophysically characterized utilizing the patch-clamp method in whole-cell recordings to characterize properties of the channel (Nadler et al. 2001). TRPM7 is regulated via intracellular magnesium and magnesium nucleotides such as Mg•ATP. With regard to Mg\(^{2+}\) regulation of the channel, it was found that the concentration of free Mg\(^{2+}\) required to inhibit half the current (IC\(_{50}\)) was 780 µM. The study went on to investigate the role of Mg•ATP in the regulation of hTRPM7 and determined the IC\(_{50}\) to be 2 mM (Demeuse et al. 2006).

TRPM7 is considered a non-specific divalent ion channel. Previous work has shown that hTRPM7 is permeable to many different divalent ions. This is one of the defining characteristics of TRPM7. The characterization of the divalent ion permeability was performed by applying a high concentration of divalent ion to a cell during whole cell patch-clamp recording and observing the inward current during application versus the inward current in the control solution. The divalent ion permeability profile of hTRPM7 was found to be Zn\(^{2+}\) = Ni\(^{2+}\) > Ba\(^{2+}\) > Co\(^{2+}\) > Mg\(^{2+}\) > Mn\(^{2+}\) > Sr\(^{2+}\) > Cd\(^{2+}\) > Ca\(^{2+}\) listed from most permeable divalent ions to least permeable divalent ions (Monteilh-Zoller et al. 2003). hTRPM6 has a similar divalent profile with Zn\(^{2+}\) > Ba\(^{2+}\) > Mg\(^{2+}\) > Ca\(^{2+}\) > Mn\(^{2+}\) > Sr\(^{2+}\) > Cd\(^{2+}\) > Ni\(^{2+}\) with the largest difference being that in TRPM6 nickel is the least permeable divalent and calcium is no longer the least permeable (Li et al. 2006).
The common non-specific inhibitor used to inhibit hTRPM7 is 2-APB. Previously, it has been shown that 2-APB can inhibit hTRPM7 and the IC_{50} has been determined to be 178 µM. 2-APB has also been shown to affect other ion channels as well such as sister channel kinase, hTRPM6, however hTRPM6 is potentiated instead of inhibited (Li et al. 2006). A specific inhibitor of hTRPM7 has been found in a soft coral. The small molecule is known as Waixenicin A and appears to be quite specific to hTRPM7. When Waixenicin A was applied to TRPM2, TRPM4, I_{cric} and TRPM6, no effect on current was observed. Waixenicin A has been characterized in hTRPM7 and the IC_{50} has been determined to be 16 nM in the presence of 700 µM internal Mg^{2+} (Zierler et al. 2011).

TRPM7 is sensitive to changes outside of the cell such as pH and osmolarity. When the extracellular pH drops below six, TRPM7 is potentiated. The low pH is thought to cause TRPM7 to have a shift in selectivity of the channel that allows for monovalent ions to permeate the channel and compete with the divalent ions for binding sites in TRPM7 (Jiang et al. 2005). Homomeric TRPM6 channels also show increases in inward current as a response to external low pH (Li et al. 2006). TRPM7 is also sensitive to external changes in osmolarity. Increases in osmolarity inhibit TRPM7 current, which is thought to be independent of cellular membrane stretch (Bessac et al. 2007). Recent research has shown that TRPM6 is not sensitive to changes in external osmolarity (Zhang et al. 2014).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Human TRPM7</th>
<th>Human TRPM6</th>
<th>Zebrafish TRPM7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg^{2+}</td>
<td>IC_{50} 720 µM</td>
<td>IC_{50} 29 µM</td>
<td>?</td>
</tr>
<tr>
<td>Mg•ATP</td>
<td>IC_{50} 2 mM</td>
<td>No effect</td>
<td>?</td>
</tr>
<tr>
<td>Divalent Profile</td>
<td>Non-specific divalent permeability</td>
<td>Non-specific divalent permeability</td>
<td>?</td>
</tr>
<tr>
<td>2-Aminoethoxy-diphenyl borate (2-APB)</td>
<td>Non-specific inhibition</td>
<td>Non-specific potentiation</td>
<td>?</td>
</tr>
<tr>
<td>Waixenicin A</td>
<td>Inhibits</td>
<td>No effect</td>
<td>?</td>
</tr>
<tr>
<td>pH</td>
<td>Low pH potentiates</td>
<td>Low pH potentiates</td>
<td>?</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>High osmolarity inhibits</td>
<td>No effect</td>
<td>?</td>
</tr>
</tbody>
</table>

**Figure 8: Human TRPM7 and TRPM6 biophysical characteristics summary**

Summary of the known biophysical characteristics of hTRPM7 and hTRPM6 that will be tested in drTRPM7.
2. Results

*Mg\(^{2+}\) and Mg•ATP sensitivity in drTRPM7*

To assess whether the biophysical characteristics of zebrafish TRPM7 (drTRPM7) are similar to those of human TRPM7 channels (hTRPM7), we started by investigating the dose-response relationship of the sensitivity of drTRPM7 currents to various concentrations of internal free Mg\(^{2+}\) in HEK cells stably expressing drTRPM7. Figure 9A shows drTRPM7 current development over time at differing concentrations of internal free Mg\(^{2+}\). The drTRPM7 current at 200 seconds after the start of the recording was used to create a dose-response graph showing the amount of drTRPM7 current at differing concentrations of internal free Mg\(^{2+}\) to determine the concentration of internal free Mg\(^{2+}\) required to inhibit drTRPM7 current to half maximal current (IC\(_{50}\)). The IC\(_{50}\) for internal free Mg\(^{2+}\) was calculated from the dose-response curve to be 804 \(\mu\)M ± 343 \(\mu\)M (Fig. 9B). Figure 9C shows the current/voltage (I/V) relationship of drTRPM7 at different concentrations of internal free Mg\(^{2+}\). The I/V characteristics of drTRPM7 remain similar in 70 \(\mu\)M internal free Mg\(^{2+}\) and 1.27 mM internal free Mg\(^{2+}\), which showed a strong outwardly rectifying current response at positive voltages (Fig. 9C). drTRPM7 appears to have similar magnesium regulation as hTRPM7.
Figure 9: Mg\(^{2+}\) and Mg•ATP sensitivity in drTRPM7.

(A) Whole cell currents in HEK293 cells over-expressing drTRPM7 were measured with an internal solution containing varying levels of free Mg\(^{2+}\), 0, 70 µM, 410 µM, 1.27 mM, 3.19 mM with n=12, n=7, n=6, n=9 and n=6, respectively. The currents were analyzed at 80mV for outward current. (B) A dose-response was calculated with the current at 80 mV at 200 seconds into recording for each concentration of free Mg\(^{2+}\) with the hill’s coefficient was set to 1. The best fit dose response curve yielded an IC\(_{50}\) of 804 µM ± 343 µM (C) A representative I-V curve showing the drTRPM7 current at 70 µM free Mg\(^{2+}\) and 1.27 mM free Mg\(^{2+}\). (D) Whole cell currents in HEK293 cells over-expressing drTRPM7 were measure with an internal solution containing 250 µM free Mg\(^{2+}\) and varying levels of Mg•ATP, 0, 0.3 mM, 1 mM, 6 mM with n=7, n=6, n=10, n=12, respectively. (E) A dose-response was calculated with the current at 80mV at the time point 200 seconds into the recording for each concentration of Mg•ATP with a hill’s coefficient set to 1. The best fit dose response curve yielded an IC\(_{50}\) 1.16 mM ± 704 µM (F) A representative I-V curve showing the drTRPM7 current at 1 mM and 6 mM Mg•ATP with 250 µM free Mg\(^{2+}\).
hTRPM7 is also regulated by levels of Mg•ATP inside the cell. A best fit dose-response curve for intracellular Mg•ATP confirmed that drTRPM7 is inhibited by Mg•ATP similarly to hTRPM, while clamping internal Mg\(^{2+}\) at 250 µM (Fig. 9D). The drTRPM7 current at 200 seconds after the start of the recording was used to create a dose-response curve that showed the IC\(_{50}\) for Mg•ATP in drTRPM7 to be 1.16 mM ± 704 µM (Fig. 9E). The I/V curve of drTPRM7 remains qualitatively unchanged, in terms of outward rectification and reverse potential after increased Mg•ATP in the cell (Fig. 9F).

**Divalent Ion Permeability of drTRPM7**

Next, the divalent ion permeability profile of drTRPM7 was studied. Permeability to divalent ions was compared against the channel’s permeability to Ca\(^{2+}\). Ca\(^{2+}\) was used to compare the permeability of other divalent ions in drTRPM7 due to calcium being the least permeable divalent ion in hTRPM7. In Figure 10A and 10B the drTRPM7 currents were normalized to the current right before application (128 sec). Ten mM of Ni\(^{2+}\) or Zn\(^{2+}\) was more permeable through drTRPM7 than Ca\(^{2+}\). Application of 10 mM Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) all were shown to be more permeable than Ca\(^{2+}\), as suspected based on hTRPM7 divalent permeability. The figure shows a representative I/V curve of application of 10 mM Ni\(^{2+}\) with the increase in inward current at negative voltages compared to the 10 mM Ca\(^{2+}\) control (Fig. 10C). The divalent permeability of drTRPM7 from highest to lowest permeability is Co\(^{2+}\), Mg\(^{2+}\), Ni\(^{2+}\), Ba\(^{2+}\), Zn\(^{2+}\), Sr\(^{2+}\), Cd\(^{2+}\), and Ca\(^{2+}\). The change % demonstrates the change in outward current measured at +80 mV as a result of the current divalent application. The increase % denotes the increase % in inward current measured at -80mV as a result of the application of the divalent (Fig. 10D).
Figure 10: Divalent profile for drTRPM7.

(A)(B) Whole cell currents in HEK293 cells over-expressing drTRPM7 were measured with an external solution containing 10 mM Ca$^{2+}$ and after 128 seconds a solution containing 10mM of divalent was applied. The currents were normalized to 128 seconds into the recording. The divalent ions applied were Ni$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Ba$^{2+}$ and Sr$^{2+}$, with $n = 6$, $n = 6$, $n = 6$, $n = 5$, $n = 5$, $n = 10$, $n = 4$ and $n = 4$ respectively. (C) Shows a representative I/V curve of drTRPM7 current with 10 mM Ca$^{2+}$ and drTRPM7 current with 10 mM Ni$^{2+}$. (D) Shows a bar graph that represents the change (%) in outward current when the divalent was applied. The bottom bar graph shows the increase in inward current when the divalent was applied.
Pharmacological Profile of drTRPM7

The next question was whether the pharmacological profile of drTRPM7 is similar to hTRPM7. Figure 11A shows application of three different concentrations of 2-APB for the duration of 100 seconds as indicated by the black bar. The three concentrations tested were 100 μM, 300 μM and 500 μM. The currents measured at -80 mV and +80 mV were normalized to the current at 200 s, which was the start of application. drTRPM7 appeared to be potentiated by the non-specific hTRPM7 inhibitor 2-APB (Fig. 11A). Two representative I/V curves showed that the classic TRPM7 characteristics of outward rectification with a reverse potential at 0 mV are present in the drTRPM7 current. The drTRPM7 current inhibition when 100 μM 2-APB was applied (Fig. 11B) and current potentiation when 500 μM 2-APB was applied was an unexpected result as 2-APB in hTRPM7 is an inhibitor of hTRPM7 current (Fig. 11C). In contrast, using the specific inhibitor of hTRPM7, Waixenicin A, on drTRPM7 had no effect on drTRPM7 currents at 10 μM concentration, a concentration previously shown to strongly inhibit hTRPM7 currents (Fig. 11D).
Figure 11: Pharmacological inhibitors of TRPM7 effects on drTRPM7.

(A) Whole cell currents in HEK293 cells over-expressing drTRPM7 were analyzed at -80 mV and 80 mV to measure the inward and outward current and normalized to the current at 200 seconds. The concentrations of 2-APB used with 100 µM (P = 0.01), 300 µM (not significant P = .11) and 500 µM (P = 0.0055) with n=5, n=5 and n=6 respectively. (B) A representative I-V curve of the drTRPM7 current before application with the thin line and the thick line represents drTRPM7 current with 100 µM 2-APB application. (C) A representative I-V curve of the drTRPM7 current before application with the thin line and the thick line represents drTRPM7 with 500 µM 2-APB. (D) Whole cell currents in HEK293 cells over-expressing drTRPM7 analyzed at -80 mV and 80 mV showing inward and outward current with Waixenicin A application at 200 seconds.
Sensitivity of drTRPM7 to extracellular pH changes

The next step was to assess the effects of pH changes on heterologously expressed drTRPM7, as hTRPM7 channels are sensitive to low pH. In these experiments, the voltage of the plasma membrane was clamped to -80 mV and external solution with different pH values was applied for 40 s as indicated by the black bar in Fig. 12A. The inward current was acquired from the current at the time point when the voltage ramp was -80 mV. From analysis of the dose-response curve by measuring the peak inward current during the pH change and plotting the averaged values against their respective pH, an EC\textsubscript{50} of pH 3.6 ± pH .07 S.D. was revealed (Fig. 12B).

Sensitivity of drTRPM7 to osmotic changes

Human TRPM7 is inhibited by hyperosmotic conditions. To assess the sensitivity of drTRPM7 to osmotic changes, heterologously expressed drTRPM7 channels were exposed to external solutions with different osmolarities for 40 seconds using the whole-cell patch clamp configuration (Fig. 12C). Currents were allowed to develop for 200s before osmotic changes were introduced via an application pipette. Currents assessed at +80 mV were normalized to the current prior to the application, and maximal current inhibition during the application was plotted against the respective osmolarity investigated (Fig. 12D). The resulting dose-response curve revealed that hyperosmotic conditions inhibited drTRPM7 with an IC\textsubscript{50} of 655 mOsm ± 8.3 mOsm (Fig. 12D).
Figure 12: pH and osmolarity sensitivity of drTRPM7.

(A) Whole cell currents in HEK293 cell over-expressing drTRPM7 were analyzed at -80 mV for the inward current and displayed inward current during application of varying pH external solutions. The HEPES buffer was used for solutions > 6 pH and MES buffer was substituted for solutions < 6 pH. (B) A dose-response was calculated with peak inward currents during the varying pH external solution application. The best fit dose-response curve yielded an EC$_{50}$ of 3.6 pH ± 0.07 pH. (C) Whole cell currents in HEK293 cells over-expressing drTRPM7 were analyzed at 80 mV for the outward current with application of external solutions at the osmolarity of 380 mOsm, 508 mOsm, 800 mOsm, 2200 mOsm with n = 10, n = 7, n = 4 and n = 5, respectively. (D) The best fit dose-response curve for osmolarity yielded an IC$_{50}$ of 655 mOsm ± 8.3 mOsm.
3. Discussion

For this series of experiments the functional regulation of drTRPM7 was examined in comparison to that found for hTRPM7. Data analysis was performed on outward current when the outward and inward current was qualitatively similar due to the outward current being much larger and less affected by the noise of recording. The data show that Mg\textsuperscript{2+} and Mg•ATP inhibit drTRPM7 current with calculated IC\textsubscript{50} values of 804 \(\mu\text{M} \pm 343 \mu\text{M}\) and 1.16 mM ± 704 \(\mu\text{M}\), respectively (Fig. 9). These IC\textsubscript{50} values are comparable to hTRPM7 values of 720 \(\mu\text{M}\) and 2 mM, respectively (Demeuse et al. 2006), revealing similar sensitivities to the allosteric regulatory molecules Mg\textsuperscript{2+} and Mg•ATP. These results suggest that drTRPM7 is regulated in a similar manner as hTRPM7 and could be involved in magnesium homeostasis within the zebrafish. It is also particularly interesting that drTRPM7 is sensitive to Mg•ATP while hTRPM6 is not sensitive to Mg•ATP (Zhang et al. 2014). hTRPM7 contains a lysine in the alpha kinase of hTRPM7 that is involved in magnesium discrimination and is conserved in drTRPM7 that may also play a role in magnesium discrimination (Schmitz et al. 2003; Demeuse et al. 2006).

When investigating the divalent profile of drTRPM7, it was found that the divalent profile for drTRPM7 from highest to lowest was Co\textsuperscript{2+} > Mg\textsuperscript{2+} > Ni\textsuperscript{2+} > Mn\textsuperscript{2+} > Ba\textsuperscript{2+} > Zn\textsuperscript{2+} > Sr\textsuperscript{2+} > Cd\textsuperscript{2+} > Ca\textsuperscript{2+} (Fig. 10). These results indicate significant differences from that previously reported for hTRPM7 of Zn\textsuperscript{2+} = Ni\textsuperscript{2+} > Ba\textsuperscript{2+} > Co\textsuperscript{2+} > Mg\textsuperscript{2+} > Mn\textsuperscript{2+} > Sr\textsuperscript{2+} > Cd\textsuperscript{2+} > Ca\textsuperscript{2+} (Monteilh-Zoller et al. 2003). By contrast, the divalent profile for hTRPM6 was reported to be Zn\textsuperscript{2+} > Ba\textsuperscript{2+} > Mg\textsuperscript{2+} > Ca\textsuperscript{2+} > Mn\textsuperscript{2+} > Sr\textsuperscript{2+} > Cd\textsuperscript{2+} > Ni\textsuperscript{2+} (Li et al. 2006). In both drTRPM7 and hTRPM7, Ca\textsuperscript{2+} is the least permeable of the divalent ions and Mg\textsuperscript{2+} has a relatively higher permeability in drTRPM7 than in hTRPM7. The large differences between hTRPM7 and
hTRPM6 appear to exist in nickel permeability, which is much lower in hTRPM6 than hTRPM7. drTRPM7 is somewhere in the middle in regards to nickel permeability and shows drastically reduced zinc permeability. Some of these discrepancies could be explained by single amino acid differences in the pore region (Fig. 3). Some of the small amino acid changes before the EVY divalent cation filter may explain why hTRPM7, hTRPM6 and drTRPM7 have slightly different divalent profiles. Overall, these results suggest that drTRPM7 is permeable to the same divalent ions as hTRPM7, which means that drTRPM7 is a possible cellular entry point for the same divalent ions as hTRPM7.

2-APB and Waixenicin A are known inhibitors of hTRPM7. When testing these compounds, it was found that drTRPM7 was potentiated instead of inhibited by 2-APB (Fig. 11A-C). The 100 µM 2-APB concentration significantly inhibited (P = 0.01) this result is likely due to ~10% of the current coming from endogenously expressed hTRPM7, which is inhibited at concentrations of 100 µM 2-APB. Another observation that should be noted is that none of the concentrations of 2-APB used completely inhibited the current, which suggests that the inhibition recorded at 100 µM 2-APB could primarily be from endogenous hTRPM7. The 500 µM 2-APB application showed significant potentiation (P = 0.0055). This is a particularly interesting result because human TRPM6 as well as hTRPM7/hTRPM6 complexes show potentiation via 2-APB application at 500 µM 2-APB (Li et al. 2006). The mechanism of 2-APB potentiation in drTRPM7 appears to behave more like human TRPM6 or hTRPM7/hTRPM6 complexes, suggesting that drTRPM7 may share some similarity with human TRPM6 as well. An alignment performed on drTRPM7 and hTRPM6 showed 49% sequence identity, as previously shown (www.uniprot.org accessed 11/14/14) (Fig. 3). Furthermore, Waixenicin A, a specific hTRPM7 inhibitor that does not affect hTRPM6, also had no effect on drTRPM7.
current. These data indicate that the pharmacological profile of drTRPM7 is similar to hTRPM6 or hTRPM6/hTRPM7 complexes, and not hTRPM7, in regards to 2-APB and Waixenicin A inhibition (Zhang et al. 2014). Therefore, drTRPM7 has a vastly different pharmacological profile than hTRPM7. This could be explained by an unknown sequence homology between drTRPM7 and hTRPM6 that doesn’t exist in hTRPM7.

Human TRPM7 is modulated by both external pH and osmolarity (Li et al. 2006; Chokshi et al. 2012; Bessac et al. 2007). The current study showed that drTRPM7-inward current was activated by lower pH. The EC$_{50}$ for current activation by pH in drTRPM7 was 3.6 pH ± .07 pH, trending toward that of hTRPM7 EC$_{50}$ of 4.7 (Li et al. 2006). This phenomenon could be explained by the conserved divalent cation filter sequence “EVY” that exists in drTRPM7, hTRPM7 and hTRPM6, which are all activated by low pH (see Fig. 3). hTRPM7 containing a point mutation at glutamate in the 1047 position loses the ability to activate in low pH conditions (Li et al. 2007). The activation of drTRPM7 current begins below pH of 5 and increases sharply at 3.4 pH, which suggests that the negatively charged glutamate may be losing the negative charge at low pH’s, which could affect the permeability of monovalent ions such as protons. The outward currents were potentiated in higher pH conditions with the exception of 3.4 pH, which had a moment of potentiation that was lost over time. The inactivation over time in the outward current may also be linked to the loss of the negative charge on the glutamate in the divalent cation filter. These results suggest that drTRPM7 may become permeable to monovalent ions that can compete for binding sites with divalent ions when the external pH drops.

drTRPM7 outward current was inhibited by hyper-osmotic conditions with an IC$_{50}$ for drTRPM7 of 655 mOsm ± 8.3 mOsm. hTRPM7 is slightly more sensitive to hyper-osmotic
changes with an IC$_{50}$ of 430 mOsm, as is hTRPM6/hTRPM7 heteromer with an IC$_{50}$ of 460 mOsm (Zhang et al. 2014; Bessac et al. 2007). Therefore this result suggests that like hTRPM7, drTRPM7 is also sensitive to osmotic pressure and could adjust channel activity in the response to external osmolarity changes.

4. Prospective Experiments

hTRPM7 was shown to be inhibited by 2-APB through an intracellular acidification mechanism (Chokshi et al. 2012). The results of the current study indicated that drTRPM7 is not inhibited by 2-APB and instead potentiated, similarly to hTRPM6 (Li et al. 2006). To investigate why drTRPM7 is not inhibited by 2-APB, a few more experiments would need to be performed.

The first experiment that could be performed to elucidate this result would be to create a chimera drTRPM7/hTRPM7 channel. Using restriction enzymes to cut sections of the aligned hTRPM7 and drTRPM7 and ligate together a full channel could be used to create the chimera channel. If the resulting channel is functional, one could then apply 2-APB to the different chimera channels and see if the chimera drTRPM7/hTRPM7 is inhibited or potentiated by 2-APB. Once it is determined which chimera is inhibited by 2-APB, one can determine which part of the channel is responsible for the inhibition via 2-APB. This experiment could help identify which part of drTRPM7 is responsible for potentiation via 2-APB application.

Another experiment that would help explain the mechanism of potentiation of drTRPM7 by 2-APB would be to investigate the acidification mechanism of hTRPM7 to determine if intracellular acidification plays a role in drTRPM7 potentiation. This experiment could be performed by using 10 mM HEPES in the internal solution, which is the standard amount currently used in the standard internal solutions as well as an experimental internal solution that
contains 140 mM HEPES, which would offer a much higher buffering capacity in the internal solution and resist the intracellular acidification. 500 μM 2-APB could be applied to drTRPM7 wild-type HEK293 T-REx cells, using the control internal solution containing 10 mM HEPES and using the experimental 140 mM HEPES solution and check for potentiation. If there is no longer potentiation of drTPRM7 current in the 140 mM HEPES solution, that would indicate that drTRPM7 potentiation by 2-APB requires internal acidification. Alternatively, this experiment could be performed by directly changing the pH of the standard internal solution and measuring drTRPM7 current to determine if drTRPM7 is potentiated by reduced pH.
Chapter 4: Analysis of the putative Mg and Mg-ATP regulatory sites in drTRPM7 through the use of truncation mutants

1. Introduction

TRPM7 plays a vital role in cellular magnesium homeostasis as well as sensing Mg•ATP levels of the cell. Currently, the model for regulation of hTRPM7 involves two binding domains, one for free Mg\(^{2+}\) binding and one more for Mg•ATP binding. The three truncated mutants of drTRPM7 can help investigate the different binding domains of drTRPM7. The experiments are designed to examine the sensitivity to the two regulators of hTRPM7 in drTRPM7 and the truncated mutants.

![Figure 13: Wild-type drTRPM7 and truncated mutants of drTRPM7](image)

drTRPM7 Wild-type and truncated mutants. drTRPM7 DK lacks the alpha kinase domain. drTRPM7 CCR lacks the alpha kinase domain and the linker between coiled-coil region and alpha kinase domain. drTRPM7 No CCR lacks the coiled-coil region, the linker and alpha kinase domain.

The kinase of hTRPM7 has been shown to be involved in nucleotide binding for Mg•ATP inhibition. The amino acid thought to be involved with Mg•ATP inhibition in hTRPM7 is K1648 in the kinase domain (Schmitz et al. 2003). When the amino acid K1648 is mutated to K1648R the intracellular nucleotide regulation via Mg•ATP, Mg•GTP or Mg•ITP is lost (Demeuse et al. 2006) which suggests that K1648 plays a role in nucleotide binding. The lysine
at position 1648 (K1648) is conserved between hTRPM7 and drTRPM7 in the alpha kinase domains (see Fig. 3). Autophosphorylation at the site S1511 and S1567 does not appear to affect channel function (Matsushita et al. 2005).

Truncations can also lead to inactivity of hTRPM7. A truncation of the kinase domain at amino acid 1569 was shown to reduce channel activity (Schmitz et al. 2003). A later study showed that a kinase deletion at the amino acid 1510 rescued hTRPM7 activity (Desai et al. 2012), which makes the area between amino acid 1569 and 1510 an area of interest in hTRPM7 activity.

The truncations in drTPRM7 that were provide by Dr. Andrew Scharenberg, (University of Washington Seattle Children’s research institute) laboratory are full-length drTRPM7 1774 amino acid length. Delta Kinase (DK) is truncated at amino acid 1478. Coil Coil region (CCR) is truncated at amino acid 1256 and No coil coil region (No CCR) is truncated at amino acid 1122 (Fig. 1).

2. Results

Mg•ATP sensitivity in truncation mutants of drTRPM7

To characterize the Mg•ATP sensitivity of all of the mutants, the currents of drTRPM7 were measured at different concentrations of Mg•ATP with internal free Mg²⁺ clamped at 250 μM. A dose-response was calculated with all of the mutants to show the sensitivity of the mutants to Mg•ATP in a dose dependent manner. The dose-responses showed the IC₅₀ for wild-type and mutant drTRPM7. Wild-type drTRPM7 IC₅₀ for Mg•ATP was 1.16 mM ± 704 μM. Delta kinase (DK) drTRPM7 IC₅₀ for Mg•ATP was 875 μM ± 457 μM and Coil Coil region
(CCR) drTRPM7 IC\textsubscript{50} for Mg\textbullet ATP was 221 \(\mu\)M ± 121 \(\mu\)M. The No coiled coil region (No CCR) mutant of drTRPM7 did not respond to Mg\textbullet ATP in a dose dependent manner, therefore, an IC\textsubscript{50} was not possible to calculate for the No CCR mutant (Fig. 14A). The increased current in the No CCR mutant may be due to the mutation itself or could be due to an increased level of overexpression within the cell line, we are unsure of which caused the increased current.

We then showed the current’s development over time for the different drTRPM7 mutants (WT, DK, CCR and No CCR) at the concentration of 1 mM Mg\textbullet ATP with a clamped internal free Mg\textsuperscript{2+} level of 250 \(\mu\)M. The concentration of 1 mM Mg\textbullet ATP was selected to show how each of the mutants drTRPM7 channel kinases behaved in physiological levels of internal Mg\textbullet ATP (Fig. 14B). The IC\textsubscript{50} for each of the mutants from wild-type to the shortest mutant No CCR was also graphed (Fig. 14C).

\textit{Mg\textsuperscript{2+} sensitivity in truncation mutants of drTRPM7}

The internal free Mg\textsuperscript{2+} sensitivity of drTRPM7 wild-type, DK, CCR and No CCR was also characterized. To begin the characterization of internal free Mg\textsuperscript{2+} in drTRPM7 mutants, the current of drTRPM7 and mutants at different levels of free Mg\textsuperscript{2+} was recorded. The resulting drTRPM7 mutant currents were displayed at the different concentrations of free Mg\textsuperscript{2+} to show inhibition in a dose dependent manner. A dose-response curve was fitted to the currents of each of the mutants to calculate and IC\textsubscript{50} of drTRPM7 mutants to internal free Mg\textsuperscript{2+}. The resulting IC\textsubscript{50} that was calculated for wild-type drTRPM7 was 804 \(\mu\)M ± 343 \(\mu\)M free Mg\textsuperscript{2+}. The IC\textsubscript{50} calculated for drTRPM7 DK was 330 \(\mu\)M ± 56 \(\mu\)M free Mg\textsuperscript{2+}. The IC\textsubscript{50} calculation for drTRPM7 CCR was 177 \(\mu\)M ± 14 \(\mu\)M free Mg\textsuperscript{2+} and the IC\textsubscript{50} for the No CCR mutant of drTRPM7 was calculated to be 747 \(\mu\)M ± 163 \(\mu\)M free Mg\textsuperscript{2+} (Fig. 15A).
Figure 14: Mg•ATP dose-response in truncated mutants of drTRPM7 in comparison to wild-type drTRPM7.

(A) Whole cell current in HEK293 cells over-expressing drTRPM7 wild-type, DK, CCR or No CCR were analyzed at 80 mV to examine the outward current of one of the versions of the channel expressed. A dose-response was calculated with the current at 200 seconds into the recording and the concentration of Mg•ATP of the internal solution n = 5 - 10. (B) The current development of wild-type and mutant drTRPM7 at internal 1 mM Mg•ATP. (C) The IC₅₀ of wild-type and mutant drTRPM7 with 1mM internal Mg•ATP.
The current development over time for each one of the mutants at 788 \( \mu \text{M} \) free Mg\(^{2+} \) was graphed to show the wild-type drTRPM7 and mutant drTRPM7 current at physiological internal free Mg\(^{2+} \) (Fig. 15B). The current development over time at physiological internal free Mg\(^{2+} \) appears consistent between the wild-type drTRPM7 and mutant drTRPM7. The IC\(_{50}\) values of each of the mutants with their corresponding truncation were graphed to show the effect on the sensitivity of the mutant drTRPM7 to internal free Mg\(^{2+} \) (Fig. 15C).
Figure 15: Free Mg\textsuperscript{2+} dose-response in truncated mutants of drTRPM7 in comparison to wild-type drTRPM7.

Whole cell current in HEK293 cells over-expressing drTRPM7 wild-type, DK, CCR or NO CCR were analyzed at 80 mV to examine the outward current of one of the versions of the channel expressed. A dose-response was calculated with the current at 200 seconds into the recording and the concentration of free Mg\textsuperscript{2+} in the internal solution n = 4 - 10. (B) The current development of wild-type and mutant drTRPM7 at 788 µM internal free Mg\textsuperscript{2+}. (C) The IC\textsubscript{50} of wild-type and mutant drTRPM7 with 788 µM internal free Mg\textsuperscript{2+}. 
3. Discussion

The truncations of drTRPM7 (DK, CCR and No CCR) and full-length drTRPM7 were investigated to find out how the truncations affect Mg•ATP binding and internal free Mg$^{2+}$ binding in comparison with the known sites in hTRPM7. The inward and outward currents were mirrored, suggesting the magnesium and Mg•ATP had similar effects on the inward and outward drTRPM7 current. The outward current was selected due to drTRPM7 current having the outward rectifying characteristic; the outward current is much larger than the inward current and is therefore less influenced by noise in the recording setup. The IC$_{50}$ of the drTRPM7 truncations for Mg•ATP was determined (Fig. 14A). At physiological levels of Mg•ATP the drTRPM7 truncations decreased the current as drTRPM7 became more truncated (Fig. 14B). In turn, as drTRPM7 became more truncated, the sensitivity to Mg•ATP increased. Once the truncation reached the point where the coiled-coil region was removed (No CCR), all sensitivity to Mg•ATP is lost (Fig. 14C). This result suggests that the proposed Mg•NTP (Demeuse et al. 2006) binding site for drTRPM7 lies in between amino acid 1122 and 1256, namely in the coiled coil region (CCR). The increased sensitivity to Mg•ATP as the truncation of drTRPM7 increased can be explained by the truncations allowing better access to the more magnesium-sensitive Mg•NTP coordinating site.

Mg$^{2+}$ binding and Mg•ATP binding sites are located separately and work together to regulate TRPM7 (Demeuse et al. 2006). Due to the independent regulation by Mg•ATP and Mg$^{2+}$, dose-response curves were performed and IC$_{50}$ values were calculated for internal free Mg$^{2+}$ in the drTRPM7 truncation (DK, CCR, and No CCR) and compared the IC$_{50}$ values to drTRPM7 Wild-type. The results showed that the DK and CCR truncations increased sensitivity.
to internal free Mg\(^{2+}\) (Fig. 15A). The No CCR truncation returned the internal free Mg\(^{2+}\) to similar sensitivity as the wild-type drTRPM7 (Fig. 15C). Under physiological levels of internal free Mg\(^{2+}\) the DK and CCR truncations showed much less current than the wild-type and No CCRR truncation (Fig. 15B). These results suggest that an additional Mg\(^{2+}\) binding site is upstream of amino acid 1122 due to magnesium sensitivity remaining in the No CCR truncation. The increase in sensitivity could be explained by the DK and CCR truncations allowing easier access to binding sites. The No CCR truncation returning to Wild-type Mg\(^{2+}\) sensitivity might be explained by the loss of the highly sensitive binding site that possibly not only accepts Mg-NTP but also magnesium (Demeuse et al. 2006). Thus, the data suggest that the Wild-type channel has two independent binding sites. One binding site for magnesium alone upstream of aa 1122, and one binding site for Mg-NTP and possibly magnesium itself and regulated by aa 1648.

Truncation of the TRPM7 kinase domain reveals a third, highly sensitive binding site sensitive to magnesium and NTP located between aa 1122 and aa 1478. However, this site is not able to differentiate between nucleotide species (Demeuse et al. 2006).

These reported results with drTRPM7 truncation leading to increased Mg\(^{2+}\) and Mg•ATP sensitivity are similar to the results reported in hTRPM7. Previously, it was reported that a kinase truncation performed at amino acid (aa) 1569, equivalent to the drTRPM7 DK mutant, reduced current in hTRPM7 but still remained functional (Schmitz et al. 2003). That same study later presented the model of multiple binding sites in hTRPM7 for Mg\(^{2+}\) and for Mg•ATP, located both on the kinase domain and channel. The authors suggested that the reduction in current is due to an increase in Mg\(^{2+}\) sensitivity in the kinase-truncated mutant.
A later study investigated the effects of truncation of TRPM7 at the kinase domain at aa 1599 and found that the truncation rendered TRPM7 non-functional (Matsushita et al. 2005). The study used Ca$^{2+}$ influx as well at patch clamping to confirm their results. An experiment to determine if the kinase truncation at aa 1599 resulted in a TRPM7 mutant that was trafficked to the membrane was not performed. The authors confirmed the expression levels were similar in the kinase truncation and wild-type TRPM7. The authors were unable to explain the loss of function in TRPM7 with a truncation at aa 1599 given the previous results of a reduced function TRPM7 with a truncation at aa 1569.

TRPM7 was recently shown to have an increase in TRPM7 current when cleaved by caspase-8 (Desai et al. 2012). A cleavage resistant mutant was unable to show increased TRPM7 current. These results in hTRPM7 are similar to the results in drTRPM7. Once the truncation reaches a certain point, No CCR mutant in drTRPM7, the second binding site is also removed leaving just the Mg$^{2+}$ binding site to regulate drTRPM7. These results suggest that there may be a third Mg$^{2+}$ binding site in drTRPM7 (Fig. 18).
4. Prospective experiments

The truncated mutants offer an opportunity to investigate the function of the truncations by looking for a loss of function in the truncation mutant. An interesting experiment that could be performed on the truncation mutants would be to do a divalent profile on the “No CCR” mutant. The idea behind this experiment would be to determine if the divalent selectivity is downstream or upstream of the shortest mutant. If the divalent selectivity changes on the shortest truncation mutant then a divalent profile could be performed on the next shortest mutant “CCR” to determine if the selectivity returns to wild-type drTRPM7 selectivity and determine which part of channel is responsible for the divalent selectivity. If the “No CCR” mutant does not have a change in selectivity, likely the selectivity filter of the channel is upstream of the “No CCR” mutant before amino acid 1122.

The loss of Mg•ATP sensitivity in the “No CCR” mutant is a very interesting result. To further investigate the proposed model that there are actually three binding sites in drTRPM7, a Mg$^{2+}$ binding site, a Mg•ATP binding site and a Mg•NTP binding site, the “DK” and “CCR” mutants could be used for further experimentation. The experiment would use internal Mg•GTP at the same concentrations used in the Mg•ATP dose-response to determine if “DK” and “CCR” mutants are sensitive to any magnesium nucleotide or if the mutants remain sensitive to just Mg•ATP. If there is no difference between the regulation in “DK” and “CCR” mutants via Mg•ATP and Mg•GTP then the “DK” and “CCR” mutants have lost the selectivity for Mg•ATP and that would suggest a third binding site on drTRPM7 that is not Mg•ATP specific, but instead can be inhibited by Mg•GTP as well.
Chapter 5: Initial characterization of native drTRPM7

1. Introduction

Studying drTRPM7 in a native cell line provides the opportunity to investigate drTRPM7 activity as well as magnesium homeostasis and pathology in a native context. Previous work has shown the hTRPM7 is ubiquitously expressed in all the cells that have been examined for hTRPM7 (Nadler et al. 2001; Runnels et al. 2001). Endogenously expressed drTRPM7 was investigated to take advantage of the ubiquitous expression pattern of TRPM7. Due to ubiquitous expression in hTRPM7, it was expected to find drTRPM7 in a zebrafish cell line (ZFL). The first step in the ZFL cells is to detect drTRPM7 and identify Mg$^{2+}$ sensitivity.

The prior characterization that was performed in this project investigated the biophysical characteristics of drTRPM7 with an overexpression system in a heterologous overexpression system, the HEK293 T-REx cells. Beginning characterization in an overexpression system is a good place to start because current sizes are large enough to see subtle channel characteristics that may be difficult to assess at low expression levels due to an unfavorable noise-to-signal ratio. There is some concern that changing the expression levels and expressing in non-native cells that there may impact the results. To address this concern, a cell line was selected that endogenously expressed drTRPM7 at physiological expression to verify magnesium regulation and potentiation by 2-APB.

2-APB has been shown to inhibit hTRPM7 and potentiate hTRPM6. 2-APB has been used in the past to distinguish between hTRPM7 current and hTRPM6 current (Li et al. 2006). An intracellular acidification is the mechanism for inhibition by 2-APB on the hTRPM7 channel.
(Chokshi et al. 2012). Our previous results in the inducible overexpression HEK293 system show that drTRPM7 is not inhibited by 2-APB and instead, is potentiated by 2-APB. An endogenous expression system was used to verify drTRPM7 current and then apply 2-APB to check for inhibition of drTRPM7 current or potentiation of drTRPM7 current. These experiments aim to provide insight into the biophysical characteristics of drTRPM7 expressed endogenously in zebrafish cells.

2. Results

*Mg^{2+} sensitivity in endogenously expressed drTRPM7*

To isolate the drTRPM7 current, whole-cell patch clamping was the selected configuration for the ZFL cells. The current development over time was graphed with varying levels of free Mg^{2+}, from zero Mg^{2+} that has been buffered with 10 mM EDTA to 3.19 mM Mg^{2+}. Without free Mg^{2+} present, the drTRPM7 current developed in ZFL cells similarly to the HEK293 T-REx overexpression system used. The current developed without free Mg^{2+} present and in the presence of 3.19 mM Mg^{2+} the current was inhibited (Fig. 16A).
Figure 16: Free Mg$^{2+}$ and 2-APB sensitivity in endogenously expressed drTRPM7.

(A) Whole cell currents from ZFL cells endogenously expressing drTRPM7 are analyzed at -80 mV and 80 mV to measure the inward and outward current. The internal solution contained either EDTA and 0 free Mg$^{2+}$ or 3.19 mM free Mg$^{2+}$ with n = 4 and n = 5 respectively. (B) Whole cell currents from ZFL cells endogenously expressing drTRPM7 are analyzed at -80 mV and 80 mV to measure the inward and outward current. 500 µM of 2-APB was applied to the cell at 400 seconds until 500 seconds into the recording n = 4 P = .069. (C) Representative I-V curve of the drTRPM7 current before application, during application of 500 µM 2-APB and after application.
2-APB sensitivity in endogenously expressed drTRPM7

2-APB was applied at the concentration of 500 µM to fully developed drTRPM7 current in the ZFL cells. The current development of drTRPM7 in the presence of 500 µM 2-APB was potentiated (although, not significantly p = 0.069) during the 100 second application and then was reversed after the application stopped (Fig. 16B). A current / voltage relationship (I/V) shows that the current recorded in the ZFL cells has the characteristic of drTRPM7 current, outwardly rectifying current with a reverse potential that passes through 0 mV (Fig. 16C).

3. Discussion

To confirm the presence of drTRPM7 currents in ZFL cells, EDTA was added to the internal solution to chelate Mg^{2+} and ensure maximum drTRPM7 current. drTRPM7 current was recorded in the ZFL cells; the current activated over 500 seconds in the presence of intracellular EDTA and zero Mg^{2+}. To determine if the drTRPM7 current is sensitive to free Mg^{2+}, 3.19 mM free Mg^{2+} was perfused into the ZFL cells. The ZFL current was inhibited when 3.19 mM free Mg^{2+} was perfused into the cell. These results together suggest that drTRPM7 current can be recorded in ZFL cells and that the drTRPM7 current recorded in ZFL cells can be inhibited by free Mg^{2+} (Fig. 16A).

The inducible overexpression system showed that drTRPM7 responds to 2-APB in an unexpected way in that drTRPM7 was potentiated by 2-APB instead of inhibited, like hTRPM7. To verify this result in a native system, 500 µM 2-APB was applied to fully developed endogenously expressed drTRPM7 current in ZFL cells; which was the concentration that was found to potentiate drTRPM7 current the most in the inducible overexpression model. The drTRPM7 current in ZFL cells was also potentiated after application of 500 µM 2-APB, which
supports the data that was collected using the inducible overexpression system in HEK293 cells (Fig. 14B). Applying 500 µM 2-APB and recording a potentiation (not significant p = 0.069) in drTRPM7 current in ZFL cells suggests that 2-APB has a similar effect in endogenously expressed drTRPM7 as in the overexpressed drTRPM7 in HEK293 T-REx cells. The lack of significance could be due to the low number of cells that could be recorded. This result could be explained by some of the sequence differences between hTRPM7 and drTRPM7. More experimentation is required to better explain why drTRPM7 is potentiated by 2-APB instead of inhibited like hTRPM7 is inhibited by 2-APB. Perhaps, one of the areas of sequence homology in drTRPM7 is more similar to hTRPM6, which causes the potentiation. Another possibility is that drTRPM7 behaves similarly to hTRPM6/hTRPM7 complexes and that results in the potentiation by 2-APB.

The current / voltage relationship of drTRPM7 before application of 500 µM 2-APB, during application of 500 µM 2-APB and after application during the washout phase showed unchanged qualitative characteristics. The current showed a strong outward rectification and a reverse potential of 0 mV with a curve that suggests an Mg²⁺ block, which is characteristic of TRPM7 (Fig. 16C). Prior to application, during application and after application the current / voltage curve shows TRPM7 characteristics, which suggests that the predominant current recorded in the ZFL cells is indeed drTRPM7 and that the application of 500 µM 2-APB is not activating another current that may account for the increase in current recorded during application. Overall, these results suggest that the endogenous expression of drTRPM7 in a native cell line shows differing pharmacology than hTRPM7. If further experimentation suggests that drTRPM7 behaves similarly to hTRPM7/hTRPM6 complexes in regards to 2-APB
inhibition, then drTRPM7 may be a good model to study hTRPM7/hTRPM6 complex related pathologies such as hypomagnesemia.

4. Prospective experiments

The more common hTRPM7 inhibitors failed to inhibit drTRPM7 and in the case of 2-APB application, a potentiation of drTRPM7 current was recorded. These results suggest that there isn’t yet a way to pharmacologically inhibit drTRPM7. The ZFL cells would be a good model to screen for a pharmacological inhibitor of drTRPM7. The ZFL cell’s endogenous expression system would give a better chance that if a natural product assay screen found a possible inhibitor that the results would translate well through the use of a cell line from the animal model the inhibitor would be used on.

In order to find a specific inhibitor for zfTRPM7, a natural product library screen could be performed using the ZFL cells in a manganese-quench assay. Inward flux of Mn\(^{2+}\) through the channel can be detected by Fura-2, a fluorescent calcium indicator, as a drop in baseline fluorescence. Fura-2 is loaded into the cells in membrane-permeable acetoxy methyl ester form, where it is cleaved by cellular esterases and hence trapped. Identification of a specific inhibitor for drTRPM7 would then allow for studies of the functional roles for the channel in neuronal cells in the zebrafish, such as in Mg homeostasis.

It would also be interesting to do a full dose-response of 2-APB in ZFL cells to determine if there is a concentration of 2-APB that inhibits drTRPM7. In the overexpression system, the inhibition recorded was thought to be due to endogenous hTRPM7 expression, which would not be an issue in ZFL cells.
Chapter 6: Conclusions

The ubiquitously expressed TRPM7 appears to have a central role in Mg$^{2+}$ homeostasis and has been shown to be involved in many diseases. Human and mouse TRPM7 share many similar biophysical characteristics and are virtually indistinguishable. Knockout of TRPM7 in a mouse model is embryonically lethal. By contrast, zebrafish can survive a loss of function mutation in TRPM7; however, there are skeletogenesis defects and melanocyte death. drTRPM7 only shares 72% sequence identity with human TRPM7. Here, the biophysical characteristics of drTRPM7 was studied in an tetracycline-inducible overexpression system in HEK293 T-REx cells, as well as endogenous expression in ZFL cells to determine whether zebrafish might represent a suitable model to study TRPM7’s involvement in development.

The study investigated hTRPM7 regulators, free internal Mg$^{2+}$ and Mg•ATP sensitivity in drTRPM7. Mg$^{2+}$ and Mg•ATP were both shown to regulate drTRPM7 similarly to hTRPM7. hTRPM7 is permeable to many different divalent cations. drTRPM7 is also permeable to the same divalent cations as hTRPM7 but to a varying degree. Specifically there were differences in zinc permeability between drTRPM7 and hTRPM7. hTRPM7 is highly permeable to zinc, where as drTRPM7 is not. The pharmacology of drTRPM7 was also invested to determine if pharmacological inhibition was suitable in drTRPM7 similarly to hTRPM7. The pharmacological inhibition of drTRPM7 was shown to be quite different than hTRPM7. 2-APB, a common non-specific inhibitor of hTRPM7, and Waixenicin A, a specific inhibitor of hTRPM7, were tested on drTRPM7. 2-APB did not inhibit drTRPM7 and instead potentiated drTRPM7, which is similar to the activity in hTRPM6, a closely related channel kinase in the TRPM family. Waixenicin A had no effect on the drTRPM7 current. Together, the 2-APB and
Waixenicin A results suggest that the pharmacological profile of drTRPM7 is similar to hTRPM6. Further experimentation will need to be performed to determine in what way that drTRPM7 and hTRPM6 may be similar. hTRPM7 is sensitive to changes in osmolarity and external pH changes. Experiments were performed on drTRPM7 to determine if drTRPM7 is similarly sensitive to osmolarity and pH changes. drTRPM7 is potentiated by low pH similarly to hTRPM7; however, drTRPM7 appears to be slightly less sensitive to osmolarity changes than hTRPM7. The biophysical characteristics of drTRPM7 seem to be very similar to hTRPM7 with the exception that the pharmacological inhibition is quite different, and indeed very similar to the pharmacological profile of hTRPM6 or that of hTRPM7/hTRPM6 complexes.

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>HUMAN TRPM7</th>
<th>HUMAN TRPM6</th>
<th>ZEBRAFISH TRPM7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg^{2+}</td>
<td>IC_{50} 720 µM</td>
<td>IC_{50} 29 µM</td>
<td>IC_{50} 804 µM</td>
</tr>
<tr>
<td>Mg•ATP</td>
<td>IC_{50} 2 mM</td>
<td>No effect</td>
<td>IC_{50} 1.4 mM</td>
</tr>
<tr>
<td>Divalent Profile</td>
<td>Non-specific divalent permeability</td>
<td>Non-specific divalent permeability</td>
<td>Non-specific divalent permeability</td>
</tr>
<tr>
<td>2-Aminooxydiphenyl borate (2-APB)</td>
<td>Non-specific inhibition</td>
<td>Non-specific potentiation</td>
<td>Non-specific potentiation</td>
</tr>
<tr>
<td>Waixenicin A</td>
<td>Inhibits</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>pH</td>
<td>Low pH potentiates</td>
<td>Low pH potentiates</td>
<td>Low pH potentiates</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>High osmolarity inhibits</td>
<td>No effect</td>
<td>High osmolarity inhibits</td>
</tr>
</tbody>
</table>

**Figure 17: Summary of biophysical characteristics of TRPM7**

Three truncated mutants were used to investigate free internal Mg^{2+} binding and Mg•ATP binding in drTRPM7. Previous models suggest separate binding sites for Mg^{2+} and Mg•ATP on
the TRPM7 channel and kinase domain. Truncation of the alpha kinase in drTRPM7 increased sensitivity to Mg\(^{2+}\) and Mg•ATP. The shortest truncation of drTRPM7, the No CCR truncation, returned the Mg\(^{2+}\) sensitivity to the non-truncated drTRPM7 Mg\(^{2+}\) sensitivity levels. The No CCR truncation also lost sensitivity to Mg•ATP. A new model is proposed where the Wild-type TRPM7 channel has two independent binding sites. One binding site for magnesium alone upstream of aa 1122, and one binding site for Mg-NTP and possibly magnesium itself and regulated by aa 1648. Truncation of the TRPM7 kinase domain reveals a third, highly sensitive binding site sensitive to magnesium and NTP located between aa 1122 and aa 1478. It is postulated that site is not able to differentiate between nucleotide species based on previous work (Demeuse et al. 2006). This model is supported by the complete loss of Mg-ATP sensitivity in the mutant missing the coiled coil region and the kinase domain, while magnesium sensitivity is preserved at various degrees in all mutants investigated.
Figure 18: drTRPM7 three binding site model: Model that contains three binding sites regulating channel activity, a Mg$^{2+}$ binding site, a Mg•ATP binding site and a Mg•NTP binding site that is more sensitive than the Mg•ATP binding site.

Endogenous expression of drTRPM7 in zebrafish liver cells (ZFL) was detected in the presence of EDTA and 0 Mg$^{2+}$. ZFL cells were also used to verify 2-APB potentiation of drTRPM7 in a zebrafish cell line. 2-APB was applied to developed drTRPM7 current and a potentiation in drTRPM7 current was recorded. The potentiation was similar to the potentiation recorded in the HEK293 cells that overexpressed drTRPM7. Endogenously expressed drTRPM7
retains a pharmacological profile that resembles human TRPM6 or TRPM6/TRPM7 complexes, rather human TRPM7 protein.

Animal model studies in zebrafish to study TRPM7 related diseases and developmental effects should be performed with an understanding that drTRPM7 behaves, in some ways, very differently than human TRPM7. However, there are currently protocols that utilize zebrafish as a model for hypoxic-ischemic brain damage that could provide useful information about drTRPM7 involvement in delayed neuronal death (Yu and Li 2011). The pharmacological studies suggest that drTRPM7 may share some similarity with human TRPM6 or TRPM6/TRPM7 complexes as well, which opens up opportunities to use zebrafish as an animal model to study TRPM6/TRPM7 complexes as well and their effect on systemic magnesium homeostasis.
References:


Li, Mingjiang, Jianyang Du, Jianmin Jiang, William Ratzan, Li-Ting Su, Loren W. Runnels, and Lixia Yue. 2007. “Molecular Determinants of Mg2+ and Ca2+ Permeability and pH


Nadler, Monica J. S., Meredith C. Hermosura, Kazunori Inabe, Anne-Laure Perraud, Qiqin Zhu, Alexander J. Stokes, Tomohiro Kurosaki, et al. 2001. “LTRPC7 Is a Mg·ATP-Regulated


Ryazanova, Lillia V., Maxim V. Dorovkov, Athar Ansari, and Alexey G. Ryazanov. 2004. “Characterization of the Protein Kinase Activity of TRPM7/ChaK1, a Protein Kinase


Zhang, Jing, Fengbo Zhao, Yin Zhao, Jing Wang, Lei Pei, Ning Sun, and Jing Shi. 2011. “Hypoxia Induces an Increase in Intracellular Magnesium via Transient Receptor Potential Melastatin 7 (TRPM7) Channels in Rat Hippocampal Neurons in Vitro.” Journal of Biological Chemistry 286 (23): 20194–207. doi:10.1074/jbc.M110.148494.

